THE ROLES OF RAC1 AND SYNCOLLIN IN REGULATED EXOCYTOSIS: INSULIN-SECRETING INS-1 CELLS AS A MODEL

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Publications

Journal Articles:

1. Amin, R., Chen, H.Q., Veluthakal R, Silver RB**, Jingsong Li,** GuoDong Li and Kowluru, A. (2003) Mastoparan-induced insulin secretion from insulin-secreting clonal β [βTC3 and INS-1] cells: Evidence for its regulation via activation of Rac, a small molecular weight GTP-binding protein. ***Endocrinology*** 144, 4508-4518

2. **Jingsong Li**, Ruihua Luo, Anjan Kowluru and GuoDong Li. (2004). Novel regulation by Rac1 of glucose and forskolin induced insulin secretion in (INS-1) β-cells. ***American Journal of Physiology – Endocrinology & Metabolism*** 286, E818-827

3. **Jingsong Li**, Ruihua Luo, ShingChuan Hooi and Guodong Li. Expression of syncollin in INS-1 β-cells impaired insulin secretion induced by glucose and other secretagogues: An essential role of its N-terminal hydrophobic sequence. Submitted to ***Biochemistry (in revision)***

Conference Papers:

1. **Jingsong Li**, Ruihua Luo and GuoDong Li. Inhibition of insulin secretion by the inhibitor of protein kinase A, H-89, mainly by a blockage of calcium channels. (Paper presented at The American Diabetes Association 60th Scientific Sessions, 9-13 June 2000, Gonzalez Convention Center, San Antonia, Texas, US). The Abstract was published in ***Diabetes***, 49, Supplement 1 (2000): A418.

2. **Jingsong Li**, Ruihua Luo and GuoDong Li. Involvement of the small G-protein Rac1 in glucose and forskolin induced insulin secretion in islet (INS-1) beta-cells. (Paper presented at The 37th Annual Meeting of the European Association for the Study of Diabetes, 9-13 September 2001, SECC, Glasgow, UK). The Abstract was published in ***Diabetologia***, 44, Suppl. 1 (2001): A62.

3. **Jingsong Li**, Ruihua Luo and GuoDong Li. Expression of a secretory granule associated protein (syncollin) affects regulated insulin secretion in INS-1 cells. (Paper presented at 62nd Scientific Sessions of American Diabetes Association, 14-18 June 2002, The Moscone Center, San Francisco, CA, US). The Abstract was published in ***Diabetes***, 51, Suppl. 1 (2002): A596.

4. **Jingsong Li**, HUO J, Luo RH and Li GD. Role of the small G-protein Rac1 in cell growth and insulin secretion in islet (INS-1) beta-cells. (Paper orally presented at Research Symposium on Islet Biology, 25-28 October 2002, Sea Crest Resort, N. Falmouth, MA, United States). The Abstract was published in ***Research Symposium on Islet Biology***, edited by American Diabetes Association, pp. 68. N. Falmouth, MA, 2002

5. **Jingsong Li**, Luo RH, Kowluru A and Li GD. Involvement of Rac1, a Small G-Protein, in Islet beta-Cell Growth and Insulin Secretion. (Paper presented at American Diabetes Association 63rd Scientific Sessions, 13-17 June 2003, New Orleans, United States). The Abstract was published in ***Diabetes***, Suppl., 52 (2003): A372

6. Amin R, Chen HQ, **Jingsong Li**, Li GD and Kowluru A. Novel roles for Rac in mastoparan-induced insulin secretion. (Paper presented at American Diabetes Association 63rd Scientific Sessions, 13-17 June 2003, New Orleans, LA, US). The Abstract was published in ***Diabetes***, Suppl., 52 (2003): A370

Summary

Regulated exocytosis, as exampled in insulin secretion stimulated by glucose and other secretagogues from pancreatic islet β cells, is regulated by multiple signaling pathways. In this study, the possible roles of two proteins (Rac1 and syncollin) in regulated exocytosis were investigated by using insulin-secreting INS-1 cells as a model system.

Rac1 is a member of the Rho family GTPases regulating cytoskeletal organization, and recent evidences implicated Rac1 in the exocytotic process. Herein, the translocation of Rac1 from the cytosol to the membrane fraction (including the plasmalemma), an indication of Rac1 activation, was found in insulin-secreting INS cells upon the exposure to the stimulatory glucose concentrations. Time course study indicated that such an effect was demonstrable only after 15 min stimulation with glucose. Furthermore, glucose stimulation increased Rac1 GTPase activity. The expression of a dominant inhibitory Rac1 mutant (N17Rac1) abolished glucose-induced translocation of Rac1, and significantly inhibited the insulin secretion stimulated by glucose and forskolin. This inhibitory effect on glucose-stimulated insulin secretion was more obvious in the late phase of secretion. However, N17Rac1 expression did not significantly affect insulin secretion induced by high K^+. INS-1 cells expressing N17Rac1 also displayed significant morphological changes and disappearance of F-actin structures. The expression of wild type Rac1 or a constitutively active Rac1 mutant (V12Rac1) did not significantly affect either the stimulated insulin secretion or the basal release, suggesting that Rac1 activation is essential, but not sufficient, for evoking secretory process. These data have demonstrated, for the first time, that Rac1 may be involved in glucose and forskolin stimulated insulin secretion, possibly at the

level of recruitment of secretory granules through regulating actin cytoskeletal network reorganization.

This study also investigated the role of syncollin, a secretory granule associated protein with possible capablility of interaction with syntaxin in a Ca^{2+}-dependent manner *in vitro*, in regulated exocytosis in the intact cell *in vivo*. To this aim, syncollin and a truncated form of the protein (without N-terminal hydrophobic domain) were stably transfected in insulin-secreting INS-1 cells that appear not to express the protein *per se*. Both the subcellular fractionation analysis and the double immunofluorescence staining revealed that the transfection of syncollin produced strong signals in the insulin secretory granules, whereas the product from transfecting with the truncated syncollin was predominantly associated with the Golgi apparatus and partly with ER. Importantly, the insulin secretion stimulated by glucose and other secretagogues was impaired in the cells expressing syncollin, but not affected by expressing the truncated syncollin. These findings have indicated that syncollin can be sorted into insulin secretory granules specifically and impair regulated insulin secretion. The N-terminal hydrophobic domain of syncollin is essential to accomplish these processes.

Abbreviations

AMP	Adenosine 3’- monophosphate
ATP	adenosine triphosphate
cAMP	Adenosine 3',5'-cyclic monophosphate, cyclic AMP
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleotide acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol-bis [-aminoethyl ether]-N, N, N’N’-tetraacetic acid
FITC	fluorescein-5-isothiocyanate
GAP	GTPase-activating proteins
GDI	guanine nucleotide dissociation inhibitors
GEF	guanine nucleotide exchange factors
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HEPS	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HRP	horseradish peroxidase
IDDM	insulin-dependent diabetes mellitus
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NIDDM	noninsulin-dependent diabetes mellitus
NSF	N-ethylmaleimide-sensitive fusion protein
PAGE	polyacrylamide gel electrophoresis

PAK	p21-activated kinase
PBS	phosphate-buffered saline
PKA	cAMP dependent protein kinase
PIP	phosphatidylinositol phosphate
PIP_2	phosphatidylinositol-4,5-diphosphate
PMSF	phenyl methyl sulforyl fluoride
RNA	ribonucleotide acid
SDS	sodium dodecyl sulphate
SNAP	soluble NSF attachment protein
SNARE	soluble NSF receptors, soluble NSF attachment protein receptors
TBS	Tris-buffered saline
TEMED	tetramethylethylenediamine
TRITC	tetramethyl rhodamine isothiocyanate

Chapter 1

Introduction

1.1 Regulation of secretory granule exocytosis

The traffic of secretory vesicles to the plasma membrane in eukaryotic cells is essential for normal cellular function. It forms the basis of intercellular communication in multicellular organisms through the release of a wide array of extracellularly acting molecules. All eukaryotic cells continuously insert vesicles into the plasma membrane by exocytosis, usually simultaneously secreting materials into the extracellular space (Palade, 1975). In addition, some cells perform more specialized forms of exocytosis that are used to release materials in a highly regulated manner. The fundamental pathway and the basic machinery for regulated and constitutive exocytosis are similar, but their regulation differs (Burgess and Kelly, 1987). The major difference between the two types of exocytosis is that, in the regulated exocytosis, secretory materials are stably accumulated in secretory vesicles or granules as storage sites, whereas in constitutive exocytosis, secretory materials are continuously released. Thus, in the regulated pathway, exocytosis of secretory vesicles is arrested at a late step and only proceeds when the appropriate stimulus is applied. A typical example is the pancreatic β-cell, which is loaded with innumerable granules containing insulin, ready to be stimulated for exocytosis when blood glucose levels rise. The regulated exocytosis has been extensively studied in synapses where it is the mechanism by which neurotransmitters are very rapidly released in a controlled manner from synaptic vesicles to mediate neurotransmission (Kelly, 1993; Zucker, 1996). A wide range of non-neuronal cell types contain regulated secretory vesicles identified as dense-core

granules or secretory granules, the contents of which serve a diverse range of physiological functions. These include the cells specialized to secrete large amounts of secretory products, for example, neuroendocrine, endocrine, and exocrine cells.

A large number of the proteins involved in the control of synaptic vesicle exocytosis has been identified (Lin and Scheller, 2000; Sollner et al., 1993; Sudhof, 1995). The interactions between these proteins and the way in which a Ca^{2+} signal leads to synaptic vesicle exocytosis are known in outline (Lin and Scheller, 2000). Similar molecular events appear to underlie secretory granule exocytosis (Brumell et al., 1995; Guo et al., 1998; Lang, 1999; Pinton et al., 2002)

The secretory granules and their regulated exocytosis have been most extensively studied in a few cell types chosen either as the model systems due to certain experimental advantages, or by their crucial physiological/pathophysiological interest. The pathway followed by secretory proteins through the cell was delineated in classical studies by George Palade in pancreatic exocrine cells (Palade, 1975). The pancreatic β-cell (Lang, 1999; Wollheim et al., 1987) is studied due to the importance of insulin secretion, and its dysfunction in both type 1 and type 2 diabetes mellitus. Haematopoietic cells, including mast cells, platelets and neutrophils (Brown et al., 1998; Chatah and Abrams, 2001; Rosales and Ernst, 2000), and adrenal chromaffin cells (Gandia et al., 1997; Vitale et al., 2000), are also widely used models for investigation of exocytosis.

1.2 Insulin secretion as a model system for studying regulated exocytosis

The pancreatic islet β-cell is a typical example of peptide-secreting endocrine cells. Proinsulin, the precursor of insulin, is synthesized in the endoplasmic reticulum and

undergoes a series of maturation steps, starting in the Golgi apparatus. The product is then packaged into secretory granules that gradually acidify, allowing further processing into insulin (Hutton, 1994). These granules are found throughout the cytosol and eventually translocated to the plasma membrane. The ultimate fusion of the granule with the plasma membrane is triggered by Ca^{2+} and controlled by a complex network of protein-protein and protein-lipid interactions that are similar in other cellular membrane fusion events, and largely conserved in eukaryotic cells. Many of the proteins involved in the regulation of neurotransmitter release have also been identified in the pancreatic β-cell and demonstrated to participate in insulin secretion (Lang, 1999)

Insulin secretion from pancreatic β-cells is a complex and precisely regulated process, constituting an important part in the regulation of body homeostasis. The secretory response in pancreatic β cells is coupled with the stimulation of glucose and other metabolizable nutrients together with hormones and neurotransmitters. Glucose and nutrients regulate insulin secretion by depolarizing the β-cell membrane resulting in Ca^{2+} influx through voltage-dependent channels, whereas hormones and neurotransmitters modulate this process by action on heterotrimeric G-proteins that transduce multiple second messengers.

Pancreatic β-cell is critical for nutrient metabolism since it is the main source to produce anabolic hormone. Therefore, the dysfunctional insulin secretion is a crucial factor in the development of diabetes, a severe metabolic syndrome characterized with hyperglycemia. Study of the exocytosis using the insulin secretion model will benefit to the understanding of both the fundamental mechanism of regulated exocytosis and the pathogenesis of diabetes development.

However, the use of primary β-cells in biochemical and molecular biology research is limited by the difficulty in isolating enough pancreatic endocrine tissue required for many basic studies on the mechanism of insulin secretion. Thus several insulin-secreting cell lines have been established, these cells retain the ability to secrete insulin in regulated manner, although their reactions to different secretagogues may vary from primary β-cells. The most widely used insulin-secreting cell lines are RIN (Gazdar et al., 1980), HIT-T15 (Santerre et al., 1981), βTC (Efrat et al., 1988), MIN6 (Miyazaki et al., 1990) and INS-1 cells (Asfari et al., 1992). These cells contain mainly insulin and in some may also have small amount of glucagon and somatostatin. RIN cells are not responsive to glucose stimulation. HIT-T15 and βTC cells secrete insulin in response to glucose but their dose-response curve is markedly shifted to the left. INS-1 and MIN6 retain the property of insulin secretion in response to the physiological ranges of glucose concentrations. In the present study, INS-1 cells were used as a model for insulin secretion.

INS-1 cells have been established from cells isolated from an X-ray-induced transplantable rat insulinoma (Asfari et al., 1992). Growth of these cells is dependent on the existence of the reducing agent 2-mercaptoethanol. The content of insulin is about 8 micrograms/10^6 cells, corresponding to 20% of the content in native β-cells. These cells synthesize both proinsulin I and II and display conversion rates of the two precursor hormones similar to those observed in rat islets although proinsulin synthesis is not stimulated by glucose. Under perifusion conditions, 10 mM glucose enhances secretion by 2.2-fold. In the presence of forskolin and 3-isobutyl-1-methylxanthine that elevate cellular cAMP levels, the increase of glucose concentration from 2.8 to 20 mM causes a 4-fold enhancement of the rate of secretion. Glucose also depolarizes INS-1 cells in a dose-dependent manner and raises the concentration of cytosolic free

Ca^{2+} ($[Ca^{2+}]_i$) between 0.5-16.7 mM (Ullrich et al., 1996). In addition, INS-1 cells have remained stable and retain a high degree of differentiation, making them a suitable model for studying various aspects of β-cell function.

1.3 Insulin secretion in normal and diabetic subjects

Insulin is an essential hormone for the maintenance of homeostasis of the blood glucose levels. The *in vivo* dose-response curve that describes the relationship between insulin secretion and glucose levels in humans is sigmoidal in shape. However, the dose-response relationship between glucose and insulin secretion is near linear when glucose levels are below 15 mM. In addition, the sensitivity of β-cells to glucose is altered by the prior exposure to glucose. Exogenous infusion of glucose increases secretion rates of β-cell upon same glucose stimulus, while a 72-hour fast causes a reduction of sensitivity of β-cells to glucose resulting in reduced insulin secretion. Low-dose glucose infusion, fasting, and refeeding can modify β-cells' response to glucose stimulation in normal weight, non-diabetic subjects (Byrne et al., 1995). The mechanism whereby changes in β-cell sensitivity to glucose are mediated has been studied *in vitro*. It has been suggested that this may involve the regulation of the enzyme glucokinase that functions as a glucose sensor, since the changes of β-cell sensitivity to glucose are correlated with alterations in the levels and activity of glucokinase (Liang et al., 1992). The activity of glucokinase in the islets plays a crucial role in glucose-induced insulin secretion, since the increased expression of the hexokinase also enhances the sensitivity of β-cell to glucose (Becker et al., 1996). Diabetes mellitus is characterized by chronic hyperglycemia, which results from a failure of the body to release adequate amounts of the blood glucose-lowering hormone insulin, from the inability of the target organs to respond to insulin for

increasing uptake of glucose, or a combination of both. Diabetes is classified into two main groups: “insulin-dependent diabetes mellitus” (IDDM or type 1) and “noninsulin-dependent diabetes mellitus” (NIDDM or type 2). Type 1 diabetes is caused by autoimmune destruction of β-cells in pancreatic islets, which results in deficiency of insulin secretion. Thus these patients require insulin injection or pancreas/islets transplantation for survival. Type 2 diabetes is characterized by the inefficacy in utilization of insulin in insulin-targeted tissues while blood insulin levels usually are not low. Additionally, already at early stages of disease the normal pattern of insulin release is disturbed so that the rapid but transient initial peak of secretion in response to a glucose challenge (first phase) is absent, while a slow but sustained insulin release remains (second phase).

About 90% diabetic patients are type 2 diabetes. Multiple factors contribute to the development of type 2 diabetes, which displays heterogeneous metabolic disorders and clinical syndromes. Both secretory defects and insulin resistance occur by the time when glucose intolerance develops. The insulin secretory abnormalities in type 2 diabetes include the rise of fasting insulin levels and the loss of the first phase of insulin secretion in response to an intravenous glucose infusion. The second phase insulin release is also delayed and attenuated. In contrast to the reduced sensitivity to glucose, insulin secretory responses to the non-glucose secretagogues (such as arginine) remain relatively intact, although the potentiated glucose effect by glucagon, secretin, and isoproterenol is impaired. Because of the secretory defects associated with diabetes, it is important to understand the molecular mechanisms underlying insulin release under both normal and pathological state.

1.4 Biogenesis of insulin secretory granules

Biologically active human insulin consists of two polypeptide chains, the A chain (21 amino acids) and B chain (30 amino acids), joined by two interchain disulfide bonds. There is also an intrachain disulfide bond in the A chain. Insulin structure is highly conserved in higher vertebrate evolution (Steiner et al., 1985). Several regions, including the position of cysteins that form the disulfide bond, the N- and C-terminal regions of A chain and the hydrophobic residues at the C-terminal of B chain, are highly conserved in evolution.

Insulin is initially synthesized as preproinsulin, the precursor of insulin, which has a 24 amino acid signal peptide in the N terminal and a 31 amino acids connecting peptide (C-peptide) between the A chain and the B chain. The signal peptide's function is to facilitate preproinsulin into the rough endoplasmic reticulum (RER). While in the lumen of RER, the signal peptide is removed, and preproinsulin is converted to proinsulin (Pfeffer and Rothman, 1987). Translocation of a newly synthesized proinsulin into the RER lumen makes its entrance into the β cell's secretory pathway. The rate of proinsulin biosynthesis is controlled by many factors, including nutrients, neurotransmitters, hormones, and protein kinase activities (Campbell et al., 1982). Glucose is the most important and potent physiological regulator of proinsulin biosynthesis (Ashcroft et al., 1978). In RER, proinsulin undergoes a folding process so that the disulfide linkage between the A and the B chain of insulin are aligned. The C-peptide is believed to aid correct structure alignment in the process (Chen et al., 2002). Correctly folded proinsulin is then delivered to Golgi apparatus from RER in transport vesicles.

After having been transported to the Golgi apparatus, proinsulin accumulates in clathrin-coated regions of the trans–Golgi network (TNG), where the secretory granules are originated. The proinsulin is sorted and targeted to the regulated secretory pathway. This is a highly efficient process, with more than 99% of the newly synthesized proinsulin delivered to the secretory granule compartment (Rhodes and Halban, 1987). The proproteins destined for dense-core granules of the regulated pathway must present features allowing them to be sorted at the level of the TGN. For proinsulin, it has been shown that residues 16 (Leu) and 17 (Glu) of the A-chain and 13 (Glu) and 17 (Leu) of the B-chain serve as 'sorting domains' for correctly sorting to secretory granules. The mutants of these residues result in diversion of proinsulin to the constitutive pathway (Orci et al., 1981).

In brief, the maturation of secretory granules includes proinsulin conversion, progressive intragranule acidification, loss of clathrin coat, and formation of hexameric insulin crystal. Matured secretory granules containing insulin and C-peptide are kept in intracellular storage pools, waiting for signals to trigger their transport to the plasma membrane for exocytosis.

1.5 Physiological regulation of insulin secretion

Glucose is the primary physiological stimulus for insulin secretion and the secretory responsiveness of β-cells is set optimally for maintenance of blood glucose in the range of 5-7 mM. Glucose enters β-cells via the high Km transporter GLUT-2. The generation of metabolic coupling factors through glucose metabolism in β-cells is the central pathway of inducing insulin secretion. The probable reason for the exquisite sensitivity of β-cells to glucose lies in the presence of the low affinity glucokinase; its Km for glucose is set at 8 mM which precisely regulates glucose phosphorylation, the

first step of glucose metabolism, in the concentration ranges at which secretion is stimulated.

Apart from glucose, a number of other nutrients including other hexoses, pentoses (such as mannose), and trioses such as glyceraldehyde, are also capable of stimulating insulin secretion. They share in common a capability to enter and be metabolized by the β-cells (Rasmussen et al., 1990). Amino acids and some species of fatty acids, such as leucine, glutamine and 2-ketoisocaproic acid, can also induce insulin release. Their metabolism in β-cells, similar to glucose metabolism, may generate ATP which in turn closes ATP-sensitive potassium (K_{ATP}) channels, leading to the membrane depolarization and Ca^{2+} entry (McClenaghan et al., 1996). Basic amino acids such as arginine are able to directly depolarize β-cells, thereby facilitating Ca^{2+} entry (Sener et al., 1989).

While glucose is the major physiological insulin secretagogue, a wide variety of hormones and neurotransmitters also affect insulin secretion through endocrine, paracrine and neural mechanisms. Glucagon, glucagon-like peptide-1 (GLP-1), and Gastric inhibitory peptide potentiate insulin secretion by binding to their receptors in the β-cell membrane which activate adenylate cyclase via interaction with a stimulatory G-protein (Gs). This in turn promotes synthesis of cyclic AMP that is a positive modulator of insulin secretion (Holst et al., 1987; Lu et al., 1993). Acetylcholine and cholecystokinin potentiate insulin secretion through increasing $[Ca^{2+}]_i$ and activating protein kinase C (PKC) following G-protein mediated stimulation of phospholipase C (PLC) (Bertrand et al., 1986; Simonsson et al., 1996). Catecholamines (such as epinephrine and norepinephrine) inhibit insulin secretion in response to various stimuli by inhibiting production of cyclic AMP, reducing Ca^{2+} entry or directly interfering with exocytosis (Persaud et al., 1989). Other agents, e.g.

somatostatin (Malm et al., 1991), pancreastatin (Efendic et al., 1987) and galanin (Ahren et al., 1989), also exhibit inhibitory effects on insulin secretion in similar manners.

1.6 Intracellular signal transduction for insulin release

Secretagogues including glucose and other fuels, hormones and neurotransmitters, stimulate insulin secretion by producing intracellular signals in β-cells. An increase of $[Ca^{2+}]_i$ is the most important signal for triggering insulin secretion. Glucose metabolism results in closure of K_{ATP} channels, leading to membrane depolarization. This causes the opening of voltage-gated Ca^{2+} channels (Dukes and Philipson, 1996). Calcium entering into β-cells may activate phospholipase A2 and PLC (Lang et al., 1994; Ramanadham et al., 1996), generating arachidonic acid and inositol 1,4,5-trisphosphate (IP_3), both of which have been shown to mobilize Ca^{2+} from pools located in ER and thus further elevate $[Ca^{2+}]_i$ (Rustenbeck and Lenzen, 1992).

Insulin secretion from β-cells is under positive or negative modulation of neurotransmitters and hormones. In contrast to the action of glucose, these agents act through membrane receptors. Signal transduction is mediated by a group of membrane associated GTP-binding proteins (G-proteins). Heterotrimeric G-proteins consist of three subunits: the α, β, and γ. These proteins are signal transducers that communicate signals from many hormones, neurotransmitters, chemokines, as well as autocrine and paracrine factors. The extracellular signals are received by members of a large superfamily of receptors with seven membrane-spanning domains and G-protein activation ensues. Activation of the G-protein is initiated by inducing the exchange of GDP for GTP on the α subunit leading to conformational change with a disassociation of the heterotrimer into G_α subunit and the $G_{\beta\gamma}$ dimmer. Both the G_α subunit and the

$G_{\beta\gamma}$ dimer act on a number of effectors. The activity of heterotrimeric GTPase is terminated by the intrinsic GTPase activity of G_α subunit (Mumby, 2000). There are at least 20 known G_α, 6 G_β, and 11 G_γ subunits. On the basis of sequence similarity, the G_α subunits have been divided into several families: G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13}$ (Neves et al., 2002). The G_s activates adenylate cyclase and mediates the response of glucagons and vasoactive intestinal peptide (Gomez et al., 2002; Johansen et al., 2001). The G_i inhibits cyclase activation and is coupled to somatostatin and α2-adrenergic receptors, providing a clue to the mechanism by which these peptides inhibit insulin secretion (Ella et al., 1997; Wittpoth et al., 2000). The G_i and G_o may also modulate protein trafficking from ER to Golgi apparatus, and then to secretory vesicles or granules (Vitale et al., 1993; Vitale et al., 1994). The G_q is classically activated by calcium-mobilizing hormones and stimulates PLC-β to produce two intracellular messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers the release of calcium from intracellular stores, while DAG recruits protein kinase C (PKC) to the membrane and activates it (Gasa et al., 1999). $G_{\beta\gamma}$ dimer also plays an important role in exocytosis, since inactivation of free $G_{\beta\gamma}$ at the level of the plasma membrane completely abolishes Ca^{2+}- and GTPγS-evoked insulin release in cloned β cells (Zhang et al., 1998).

Protein phosphorylation is a common way to regulate insulin secretion. The important second messengers calcium, cAMP, and diacylglycerol, which are generated from glucose metabolism and receptor agonist stimulation, activate Ca^{2+}/calmodulin-dependent protein kinase II, protein kinase A and protein kinase C, respectively. These protein kinases phosphorylate a variety of proteins in β-cells, which participate in the cascade of stimulation of insulin secretion (Jones and Persaud, 1998).

1.7 Rho family of small GTPases as molecular switches

The Rho GTPases form a subgroup of the Ras superfamily of 20-30 kD GTP-binding proteins that have been shown to regulate a wide spectrum of cellular functions. These proteins are ubiquitously expressed from yeast to mammalian cells. Rho was first identified in 1985 as a small GTP-binding protein related to Ras (Madaule and Axel, 1985). This protein is a target of the *clostridium botulinum* C3 transferase, a bacterial coenzyme that induces ADP ribosylation (Williamson et al., 1990). Several other members of the Rho family have been identified, including Cdc42 and Rac (Shinjo et al., 1990; Shirsat et al., 1990).

Different mammalian Rho GTPases are at least 40% identical to each other at the amino-acid level, whereas they are approximately 25% identical to Ras. To date, only Rho, Rac, and Cdc42 have been characterized extensively in the Rho family. In mammals, there are three highly homologous isoforms of Rho, known as RhoA, RhoB, and RhoC, which are over 85% identical at the amino-acid level. The majority of the differences lie within the last 15 amino acids of the carboxy terminus (Ridley, 2000). Similarly, Rac1, Rac2, and Rac3 are over 88% identical, and differ primarily within the carboxy-terminal 13 amino acids (Haataja et al., 1997). Rac1 is widely expressed in different tissues and cell lines, while Rac2 is only expressed in haematopoietic cells and Rac3 appears to be expressed selectively in the developing nervous system (Haataja et al., 1997; Shirsat et al., 1990). The Cdc42 gene was initially identified in *S. cereoisiae* as a cell cycle mutant defective in budding (Johnson and Pringle, 1990). It has two mammalian isoforms with different carboxy terminal sequences (Shinjo et al., 1990).

The members of the Rho family have emerged as important players in signal transduction processes activated by a variety of both extracellular and intracellular

stimulants. These molecules are the major regulators of actin cytoskeleton in eukaryotic cells (Hall, 1998). In addition, they are also involved in many other cellular responses, including activation of MAPK cascades and regulation of transcription factors, secretion, endocytosis, cell polarity, and the cell cycle. Accordingly, they play crucial roles in the development and behaviors of multicellular organisms.

1.8 Regulation of the activity of Rho GTPase

Rho proteins are active when bound to GTP and inactive when bound to GDP. The transition of the two forms is regulated by three groups of proteins (Fig. 1.1). The guanine nucleotide exchange factors (GEFs) enhance the exchange of bound GDP for GTP, leading to activation of G-proteins. The GTPase-activating proteins (GAPs) increase the rate of hydrolysis of bound GTP, resulting in inactivation of G-proteins. The guanine nucleotide dissociation inhibitors (GDIs) bind the inactive form of G-proteins and retain them in the cytosol.

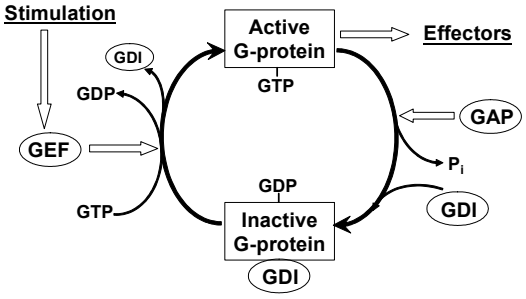

Fig. 1.1. Schematic diagram for the regulation of activity of Rho proteins. It is known that the inactive Rho proteins are associated with GDI and retained in cytosol. In response to upstream stimulating signals, GEFs activate the Rho proteins by promoting their binding to GTP and releasing GDP. The active G-proteins are then translocated from cytosol to the target membranes where they interact with their effectors that initiate downstream responses. The GTPase activity is markedly enhanced by GAP, which renders the Rho proteins inactive and facilitates their relocation to cytosol where they binds to GDI.

Over 30 potential GEFs for Rho GTPases have been identified. All possess a conserved exchange factor domain, known as the Dbl homology (DH) domain, adjacent to a pleckstrin homology (PH) domain (Van Aelst and D'Souza-Schorey, 1997). The majority of GEFs can activate several Rho GTPases *in vitro*, although some show preference for one or a subgroup of proteins. The active Rho proteins would associate with target membranes, where they interact with downstream effectors.

The intrinsic GTPase activity of Rho proteins normally is very low and requires Mg^{2+} (Zhang et al., 2000). Once Rho proteins are activated upon binding GTP, their activity is terminated by GTP hydrolysis, yielding GDP binding forms and release of phosphate. The GTP hydrolysis rate can be markedly enhanced by GAPs. The GAPs for Rho family proteins share a similar fragment of 140 amino acids, known as the RhoGAP domain, which is sufficient to confer GAP activity.

Activity of the Rho family GTPases is regulated cyclically. In the cytosol of resting cells, the GDP-bound GTPases form a complex with GDIs. It is believed that they maintain inactive Rho GTPases in the cytoplasm until an appropriate stimulus induces dissociation of the complex and concomitant exchange of bound GDP for GTP due to GEFs. *In vitro* studies have shown that binding of GDIs to Rho proteins not only prevents nucleotide exchange (thereby preventing activation) but also inhibits intrinsic and GAP-stimulated GTP hydrolysis (thereby preventing deactivation) (Chuang et al., 1993; Takaishi et al., 1993). The GDIs can also extract Rho proteins from membranes and keep them in a soluble complex in the cytoplasm by possibly masking the membrane-binding prenyl group (Sanford et al., 1995).

1.9 Constitutively active and dominant inhibitory form of Rho GTPase

The mutations of a number of conserved amino acids in Ras superfamily proteins decrease the intrinsic and GAP-stimulated GTPase activity, allowing these proteins to remain predominantly in the GTP-bound, active form. The proteins with mutations of 12^{th} amino acid from glycine to valine, or 61^{st} amino acid from glutamine to leucine (number of RhoA), are the most commonly used constitutively active forms of Rho GTPases (Fig. 1.2).

Dominant inhibitory forms of Rho proteins have been particularly useful in assessing their function. The most widely used dominant inhibitory Rho proteins are created by substituting amino acid 17 (number of RhoA) from threnine to asparagine (Fig. 1.2). The amino acid at this position is essential to coordinate with a Mg^{2+} ion required for guanine nucleotide binding in all Ras superfamily of GTP-binding proteins (Bourne et al., 1991). The substitution of this amino acid is therefore predicted to interfere with both Mg^{2+} and nucleotide binding, and indeed Rac and Cdc42 proteins with this mutation have a much lower affinity for GTP/GDP than their wild-type counterparts (Self and Hall, 1995). It is also believed that they inhibit the activity of their respective endogenous GTPases by competing for binding to GEFs (Feig, 1999).

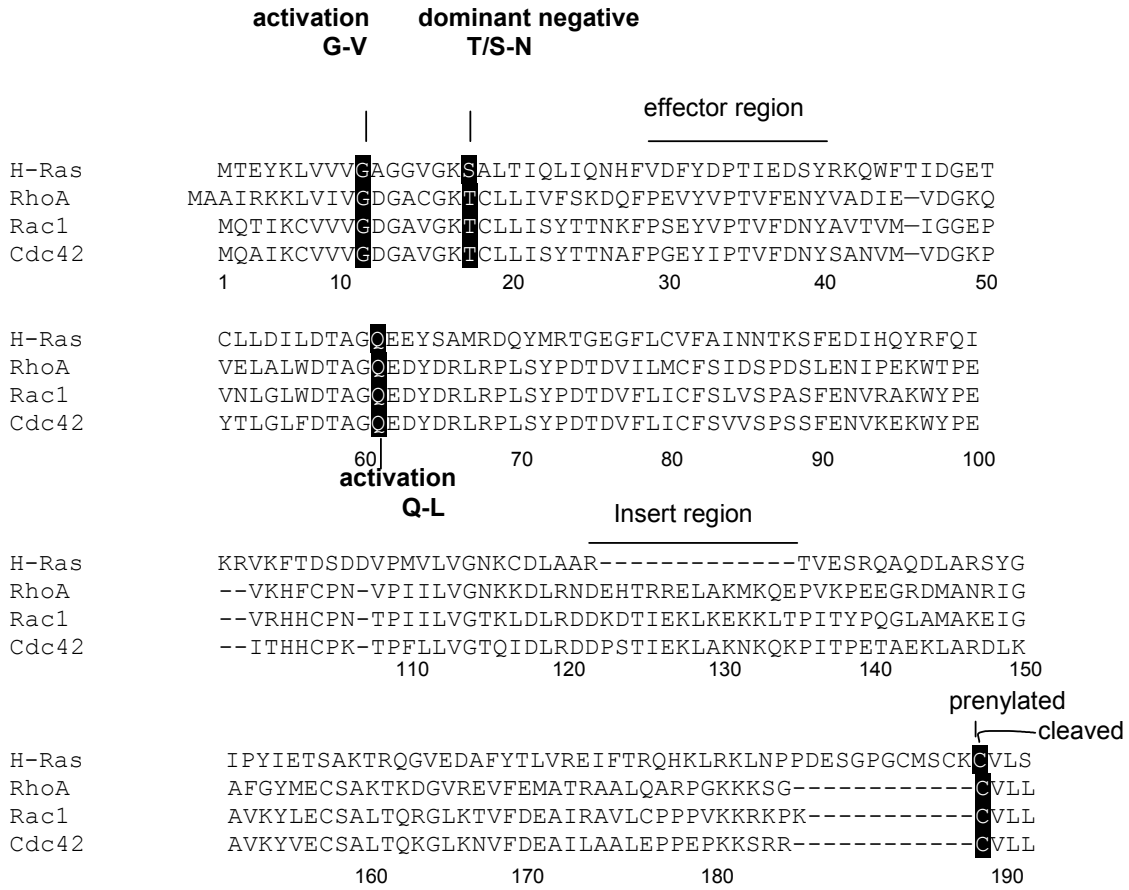

Fig. 1.2. Amino Acid sequence alignment of human H-Ras, RhoA, Rac1, and Cdc42 proteins. Common mutated amino acids for producing protein with altered behaviors are demonstrated in white letters: mutation of amino acid 12 and 61 generate GTPase-defective mutants, and mutation of amino acid 17 generates dominant inhibitory mutants. Numbers refer to RhoA. (Adopted from Ridley, 2000)

1.10 Rac-mediated cytoskeleton organization

The first characterized responses to Rho GTPases in mammalian cells link the plasma membrane receptors to the assembly and organization of the filamentous actin cytoskeleton. Rac regulates the formation of lamellipodia and membrane ruffles in a variety of cell types (Tapon and Hall, 1997). The lamellipodia are plasma membrane protrusions containing a meshwork of actin filaments. The membrane ruffles are similar in structure to lamellipodia, but protrude upwards from the dorsal

surface of adherent cells. Both lamellipodium extension and membrane ruffling require active actin polymerization at the plasma membrane.

A number of Rac effectors act as regulators of actin polymerization. The p21-activated kinase (PAK) that is activated by binding to active Rac or Cdc42, serves as a downstream effector for actin reorganization (Manser et al., 1997; Sells et al., 1997), leading to lamellipodium formation. IQGAP is another protein with potential role in cytoskeleton organization. It interacts with both Rac and Cdc42 and is localized to membrane ruffles. It appears to regulate cadherin-catenin interactions during cell adhesion and mediate actin reorganization (McCallum et al., 1998; Natale and Watson, 2002). Rac can associate with phosphatidylinositol 5-kinase (PIP5-kinase) physically *in vitro* in a GTP-independent manner (Tolias et al., 2000). A complex of Rac with a type I PIP5-kinase and diacylglycerol kinase has been purified from cells (Tolias et al., 1998). It is suggested that PIP5-kinase stimulates actin polymerization probably by removing phosphoinositide-regulated capping proteins from actin filaments (Schafer et al., 1996). POR1 (partner of Rac) was found to interact specifically with Rac in a GTP-dependent manner and play a role in Rac-mediated membrane ruffling (Van Aelst et al., 1996). Arp2/3 complex is the only known cellular factor to nucleate new actin filament, and can be activated by nucleation-promoting factors (NPFs) to initiate actin polymerization (Welch and Mullins, 2002). During the formation of lamellipodia, Rac regulates Arp2/3 activity through NPFs WAVE/Scar (Miki et al., 1998).

1.11 Rac in regulated exocytosis

The trafficking of vesicles within cells involves the interactions with the actin cytoskeleton as well as with microtubules (Kelleher and Titus, 1998). The Rho family

proteins can affect several aspects of vesicle trafficking, and the most significant role is inducing actin reorganization.

In permeabilized mast cells, recombinant Rac proteins enhance secretion induced by various agents, although they cannot stimulate secretion by themselves (Brown et al., 1998; O'Sullivan et al., 1996). Conversely, *C3* transferase and dominant inhibitory Rac inhibit secretion induced by calcium and non-hydrolysable GTPγS (O'Sullivan et al., 1996; Price et al., 1995). Rac was also purified from mast cells and neuroendocrine cells as a factor that can enhance secretion (Doussau et al., 2000; O'Sullivan et al., 1996). In natural killer cells, expression of dominant inhibitory Racl inhibit granule exocytosis (Billadeau et al., 1998). Using clostridial toxins to specifically inactivate certain members of Rho proteins in pancreatic β cells also imply that Rac and Cdc42 (rather than Rho) may be the candidate regulators implicated in stimulated insulin secretion (Kowluru et al., 1997b). However, the mechanisms underlying the involvement of Rho and Rac in exocytosis and secretion have not been established. It has been shown that phospholipids such as PIP2 and the kinases regulating their turnover serve as a link between Rho GTPases and actin cytoskeleton (Carpenter et al., 1999). Thus, it is possible that the local changes in membrane phospholipids, for example through activation of PIP5-kinase, PI3 kinase, or phospholipase D (PLD), may induce changes in actin-based cytoskeleton and regulate vesicle-target interaction (Martin, 1998).

1.12 SNAREs machinery for exocytosis

SNAREs (soluble N-ethylmaleimide-sensitive fusion protein [NSF] attachment protein [SNAP] receptors) represent a class of membrane-bound proteins that is defined by the presence of SNARE motif, a signature sequence of ~60 residues.. This motif mediates the assembly of SNAREs into an extremely stable coil-coiled core complexes during

fusion of vesicles with target membranes (Sutton et al., 1998). SNAREs are classified into two groups, the v-SNAREs and the t-SNAREs based on their localization on vesicle donor or target acceptor membranes. Another classification system is based on the conserved glutamine (Q) or arginine (R) in the SNARE motif (Fasshauer et al., 1998). In practice Q-SNAREs are usually t-SNAREs and R-SNAREs are usually v-SNAREs. A large number of SNARE proteins have been identified that act in different membrane-trafficking reactions all over the cell. Two groups of plasma membrane SNAREs (synaptosomal-associated protein-25 [SNAP-25] and syntaxins) interact with one group of vesicular SNARE (synaptobrevins, also called vesicle-associated membrane proteins [VAMP]) in exocytosis (Sollner et al., 1993). The first evidence that SNAREs, in particular the synaptic SNAREs syntaxin, SNAP-25, and synaptobrevin, are involved in membrane fusion is derived from the observation that botulinum and tetanus toxins specifically block synaptic vesicle exocytosis by attacking the synaptic SNARE proteins (Blasi et al., 1993; Schiavo et al., 1992). Each exocytotic SNARE has multiple closely related isoforms: SNAP-25 and -23; syntaxins 1, 2, 3, or 4; and synaptobrevins 1, 2 and cellubrevin. Synaptobrevin/VAMP is a short, very abundant synaptic vesicle protein (~120 residues) composed of an N-terminal 30-residue proline-rich sequence that is not well conserved between species, a central SNARE motif, and a COOH-terminal hydrophobic region (Sudhof et al., 1989; Trimble et al., 1988). SNAP-25, or synaptosomal protein of 25 kDa, is composed of two SNARE motifs that are connected by a long linker sequence containing multiple cysteine residues. SNAP-25 lacks a transmembrane region, and is attached to the membrane via multiple palmityl residues that are bound to the cysteine residues in the central region (Oyler et al., 1989). Syntaxin is similar to synaptobrevin in that it contains a COOH-terminal SNARE motif followed by a single transmembrane region

that anchors it in the membrane. Different from synaptobrevin, however, the N-terminal sequence of syntaxin is relatively long (~180 residues) and forms an independently folded three-helical domain called the Habc domain (Fernandez et al., 1998). In pancreatic β-cells, synaptobrevin, SNAP-25 and syntaxin are required in Ca^{2+}-evoked exocytosis of insulin granules (Regazzi et al., 1995; Sadoul et al., 1995; Wheeler et al., 1996). SNAP-23, which is not cleavable by botulinum toxin, can replace the function of SNAP-25 in β-cells (Sadoul et al., 1997). Synapobrevin/VAMP can also be replaced by the isoform cellubrevin (Regazzi et al., 1996b). Syntaxin synthesis is regulated by glucose stimulation (Nagamatsu et al., 1997). NSF with adaptor proteins, rab proteins, and SM proteins (Sec1/Munc18-like proteins) are other three groups of proteins that co-work with SNAREs in exocytosis regulation. NSF is an ATPase that binds to SNARE complex via adaptor proteins SNAPs (Wilson et al., 1989). It disassembles the core complexes in an ATP-dependent manner (Jahn and Sudhof, 1999). NSF is required for Ca^{2+}-stimulated insulin secretion (Kiraly-Borri et al., 1996; Vikman et al., 2003). SM proteins are soluble proteins of ~65kDa that bind to syntaxin family. There are three Munc 18 isoforms, Munc 18a, b, and c. Munc18 can bind to the closed conformation of syntaxin 1 (Dulubova et al., 1999). Therefore it may regulate the binding of syntaxins with SNAP-25 since syntaxins cannot simultaneously bind to SM proteins and other SNAREs (Pevsner et al., 1994). Rab proteins belong to another subfamily of small GTP-binding proteins that appear to be associated with specific fusion events. More than 60 Rab proteins are expressed in mammalian cells (Pereira-Leal and Seabra, 2000). Only 2 classes of Rab proteins (rab3 and rab27) with a function in endocrine and synaptic exocytosis have been identified (Darchen and Goud, 2000; Yi et al., 2002). Rab3s are primarily regulatory, and appear to play a role only in regulated exocytosis. Rab3A is associates with secretory granules

in pancreatic β cells, while Rab3-interacting molecules such as RIM are localized at the plasma membrane (Iezzi et al., 2000; Inagaki et al., 1994; Regazzi et al., 1996a). Rab3A knockout mice are glucose intolerant and display a defect in glucose-induced insulin secretion (Yaekura et al., 2003). Overexpression of wild-type Rab27a and its GTPase-deficient mutant increase evoked insulin secretion in mouse β cells (Yi et al., 2002). Silencing of Rab27a expression by RNA interference reduces the secretory capacity of β-cells (Waselle et al., 2003). Synaptotagmin is the most likely candidate for the Ca^{2+}-sensor of the exocytotic machinery (Fernandez-Chacon et al., 2001), and essential for Ca^{2+}-dependent neurotransmission and exocytosis of insulin-containing granules (Littleton et al., 1993; Lang et al., 1997). It is a protein of 65 kDa that spans the vesicle membrane once and contains a large cytoplasmic domain with two Ca^{2+}-binding C2 domains (protein kinase C-homology domains), C2A and C2B (Sutton et al., 1995). In addition to binding Ca^{2+}, the C2 domains are involved in the association of synaptotagmin with phospholipids, SNAREs, and a variety of other proteins. Several isoforms of the protein have been identified, of which synaptotagmin III and synaptotagmin VII are present in β-cells (Gao et al., 2000; Lang et al., 1997). Beside synaptotagmin, syncollin and taxlilin are also potential Ca^{2+}-dependent proteins involving exocytosis by binding with syntaxin (Edwardson et al., 1997; Nogami et al., 2003).

1.13. Aims of Studies

Although Ca^{2+} is considered as the universal link in the stimulus-secretion coupling of regulated exocytosis, there is existence of Ca^{2+}-independent, but GTP-dependent, exocytosis in both mast cells and pancreatic β-cells (Pinxteren et al., 2000; Metz et al., 1992). The concept of a G protein (GE) controlling exocytosis is postulated and well

developed although it has yet to be identified (Gomperts et al., 1986; Gomperts et al., 1990). It is believed that both heterotrimeric and monomeric GTP-binging proteins are involved in the control of exocytosis. On the other hand, it is well known that glucose stimulates insulin secretion from islet β-cells by both K_{ATP}-dependent and –independent signaling pathways; the former is mainly promoting $[Ca^{2+}]_i$ rises whereas the nature of the latter remains to be defined with the G-protein to be a candidate.

Previous studies (Kowluru et al., 1997b; Daniel et al., 2002; Kowluru et al., 2003) have provided indirect evidence for the involvement of Rho subfamily of GTP-binding proteins in physiological insulin secretion. Such evidence came from the observations that stimulation of insulin secretion could be suppressed upon general inhibition of requisite post-translational modifications (i.e., farnesylation, carboxyl methylation, and fatty acylation) of small G-proteins, and when Rho subfamily GTP-binding proteins were selectively glucosylated and inactivated by Clostridial toxins (Aktories et al., 2000; Kowluru et al., 1997b). However, Rho seemed not to play an important role in this event, since its inactivation by *botulinum toxin* C3 failed to affect stimulated insulin secretion (Kowluru et al., 1997b). In addition, it has been demonstrated that under conditions of stimulated insulin secretion, glucose augmented the carboxyl methylation and membrane-association of Rho family of GTP-binding proteins (Kowluru et al., 1997a). All these findings suggested a possible involvement of Rho GTPases (potentially Rac or/and CDC42) in insulin secretion. Therefore, one of the aims of this study was to examine the putative regulatory role for Rac1, a low molecular weight small GTP-binding protein, in physiologic insulin secretion from insulin-secreting INS-1 cells, by studying its activation under physiological

stimulation conditions and by modifying its activity through expressing of dominant inhibitory and constitutively active forms of its mutants.

Syncollin was originally cloned from pancreatic acinar cells and the synthesized GST-protein could interact with syntaxin in Ca^{2+}-dependent manner and affect exocytosis in a cell-free system (Edwardson et al., 1996), at the time when this study was initiated while a collaborator independently cloned its gene from rat duodenum (Tan and Hooi, 2000). Since this protein was found associated with zymogene granules, containing a 19-amino acid N-terminal hydrophobic domain and interacting with syntaxin (Edwardson et al., 1996), all the typical properties of a v-SNARE, it would be logical to investigate its possible physiological role in exocytosis in an *in vivo* system. Although I was unable to detect its expression in β-cells and thus syncollin might not play a physiological role in insulin secretion, it was thought that such a well-established exocytosis cell system could be useful to determine the possible function of syncollin in regulated exocytosis *in vivo* and improve the understanding of its biochemical features. To this end, the genes of syncollin and its truncated form (lack of N-terminal hydrophobic domain) were expressed in insulin-secreting INS-1 cells (not expressing syncollin) to investigate its effect on regulated exocytosis and the function of the hydrophobic sequence.

Chapter 2

Materials and Methods

2.1 Cells

Insulin-secreting INS-1 cells and INS-1 derived cells (see a table below) were grown in RPMI 1640 (Sigma) containing 10% fetal bovine serum (GIBCO BRL), 50 μM 2-mercaptoethanol and 1 mM pyruvate in culture flasks (Falcon) at 5% CO_2. For transfected cell lines, additional antibiotics were added in culture medium for cell selection.

Cells used in the study

Cell type	Origin	Passage	Culture medium
INS-1	Rat	56-76	RPMI with 50 μM 2-mercaptoethanol and 1 mM pyruvate (complete medium)
INS-1 pIRES	INS-1 Transfected with vector pIRES	62-78	Complete medium + 50ug/ml hygromycin
INS-1 N17Rac	INS-1 Transfected with vector pIRES-N17Rac1	62-78	Complete medium + 50ug/ml hygromycin
INS-1 V12Rac	INS-1 Transfected with vector pIRES-V12Rac1	62-78	Complete medium + 50ug/ml hygromycin
INS-1 pCDNA	INS-1 Transfected with vector pCDNA	62-76	Complete medium + 50ug/ml geneticin
INS-1 syncollin	INS-1 Transfected with vector pCDNA-syncollin	62-76	Complete medium + 50ug/ml geneticin
INS-1 truncated syncollin	INS-1 Transfected with vector pCDNA-truncated syncollin	64-76	Complete medium + 50ug/ml geneticin

2.2 Molecular biology

2.2.1 Buffers

Buffers	Composition
Resuspension buffer for plasmid mini Prep	50 mM Tris.Cl, pH 8.0; 10 mM EDTA; 10 μg/ml RNase A
Lysis buffer for plasmid mini Prep	200 mM NaOH, 1 % SDS
Neutralization buffer for mini Prep	3.0 M potassium acetate, pH 5.5

2.2.2 Bacterial strain

XL1 blue E. coli strains (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]) were used for amplification of plasmids.

2.2.3 Molecular cloning

The plasmids with cloned human Rac1 mutants were constructed by subcloning into vector pIREShyg1 (Clontech, Palo Alto, CA, USA) from pEXVV12rac and pEXVN17rac. N-terminals of the mutants were tagged with c-Myc as a marker which can be recognized by an anti-c-Myc antibody. (This two constructs are generous gifts from Dr. Marie-Veronique Clement, National University of Singapore). The dominant inhibitory mutant Rac1N17 has a mutated amino acid threonine 17 substituted by asparagine and the constitutively active mutant Rac1V12 has amino acid glycine 12 substituted by valine. Control vector were constructed by deleting the inserts from above plasmids by 5'BamHI and 3'NotI restriction enzyme (Promega) digestion followed by blunt ligation using DNA polymerase I large (Klenow) fragment and T4 ligase (Promega). Wild type Rac1 was cloned in to pIREShyg1 by using non-mutated forward primer followed by PCR, restriction enzyme digestion and ligation.

The two plasmids containing syncollin or its truncated form (lack of the N-terminal hydrophobic domain of 19 amino acids) were constructed by cloning them into vector pCDNA 3.1A/Myc-His (Invitrogen) by RT-PCR, in which 5'-accatgtccccgctgtgcct-3' and 5'-tcaatagcacttgcagtaga-3' were used as forward and reverse primers for syncollin while 5'-cgaggcgcttgtccagtgcc-3' and 5'-tcaatagcacttgcagtaga-3' were used as forward and reverse primers for the truncated syncollin. Empty vector was used as control plasmid. Myc-tagged syncollin was constructed by cloning syncollin in pCDNA 3.1A/Myc-His without terminal codes.

Plasmids used in the studies

Plasmid	**Description**
pIRES	Deleting the inserts from pIRES-N17Rac1
pIRES-N17Rac1	5' flank c-myc tagged N17Rac1
pIRES-V12Rac1	5' flank c-myc tagged V12Rac1
pIRES-Rac1	5' flank c-my tagged Rac1
pCDNA	pCDNA3.1A/Myc-His
pCDNA-syncollin	Syncollin was cloned in pCDNA3.1A
pCDNA-truncated syncollin	Truncated syncollin was cloned in pCDNA3.1A

2.2.4 Transformation of *E. Coli*

Purified plasmid DNA (50 ng) was added to 100 µl competent *E.Coli* cells, and mixed by swirling the tube gently followed by incubating on ice for 10 min. Cells were heat shocked by placing in 42^{o}C water bath for 90 s followed by incubation of 2 min on ice. LB medium (400 µl) was added to each tube and incubated at 37^{o}C for 1 hr on a

shaker (200 rpm). Bacterial culture (100 μl) was plated on the LB plate containing 100 μg/ml ampicillin and incubated 12-16 hrs.

2.2.5 DNA preparation

Plasmid DNA Mini preparation

Plasmid used for analysis was isolated by a modified protocol from Qiagen's mini prepare kit. Bacteria (2-5 ml) were culture in LB medium overnight. Bacterial culture (1-2 ml) was transferred to 1.5 or 2 ml tubes and centrifuged at 11,000 *g* for 30 s. Supernatants were removed and the bacterial pellets were resuspended in 0.3 ml of resuspension buffer with RNase A. The bacteria should be resuspended completely, leaving no cell clumps. Afterwards, 0.3 ml of lysis buffer was added to the tubes, which were mixed gently by inverting 4–6 times and incubated at room temperature for 5 min. Thereafter, 0.3 ml of chilled neutralization buffer was added, mixed immediately but gently, followed by incubation on ice for 5 min. Lysate was centrifuged at maximum speed in a microcentrifuge for 10 min. Supernatants were transferred to new centrifuge tubes, and DNA was precipitated with 0.7 volume of isopropanol. The tubes were immediately centrifuged at 10,000 rpm for 30 min, and the supernatants were carefully decanted. DNA pellets were washed with 1 ml of 70% ethanol, air-dried for 5 min, and dissolved in a suitable volume of buffer. DNA concentrations were measured by UV spectrophotometry to determine the yield.

Plasmid DNA Midi preparation

Midi preparation of plasma DNA was carried out using Bio-Rad midi kit. Bacteria (30-50 ml) were cultured in LB medium overnight. The culture was harvest at 6,000 *g* for 15 min at 4^{o}C in Beckman Centrifuge. The pellets were resuspended by pipetting in 4 ml S1 buffer with RNase A, followed by adding 4 ml S2 buffer and mixing by

inverting 6-8 times. The tubes were then incubated 2-3 min at room temperature. Thereafter, 4 ml cold S3 buffer was added and mixed by inverting 6-8 times, followed by 5 min incubation on ice. The lysates were centrifuged at 12,000 *g* for 25 min at 4^{o}C. The clear lysates were then loaded onto columns equilibrated with 2.5 ml N2 buffer. After the lysate flowed out, the column was washed with 10 ml N3 buffer. The plasmids were eluted with 5 ml N5 buffer and precipitated by adding 3.5 ml isopropanol, followed by centrifuging at 15,000 *g* for 30 min at 4^{o}C. Subsequently, the pellets were washed with 2 ml 70% ethanol, and centrifuged at 15,000 *g* for 10 min at room temperature. Air-dried plasmids were dissolved in TE buffer and DNA concentrations were determined by UV spectro-photometry.

Purification of DNA fragments from agarose gel

The gels with DNA bands were chopped finely, followed by adding water to final volume about 300 μl. An equal volume of buffered phenol (Sigma) was added and mixed by vortex. The mixture was incubated in -80^{o}C for 15 min, and centrifuged at 14,000 rpm for 20 min at room temperature. The top aqueous layer was transferred to a new centrifuge tube and a half volume of buffered phenol and chloroform each was added, followed by centrifuging at 14,000 rpm for 5 min at room temperature. The top layer was transferred to a new centrifuge tube. One tenth volume of 3.0 M sodium acetate and 2 volume of cold pure ethanol were added, followed by incubating in -20^{o}C for 30 min and centrifuging at 14,000 rpm for 30 min at 4^{o}C. The pellets were washed with 500 μl cold 70% ethanol and dissolved in TE buffer.

Purification of DNA fragments from solution

DNA by restriction enzyme digestion was purified by using GFX DNA purification Kit (Amersham). In brief, 500 μl capture buffer was added to the reaction buffer

containing DNA and restriction enzyme and mixed with pippetting. The volume of reaction buffer was topped to 100 µl and the mixture was transferred to a DNA binding column and incubated for 1 min at room temperature. The column was centrifuged at full speed in a common centrifuge for 1 min. Thereafter, 500 µl wash buffer was added to the column that was centrifuged. DNA was eluted by adding distilled water to the column followed by centrifuging.

2.2.6 RNA purification

Cells cultured in 60-mm dishes were used to isolate total RNA by using RNeasy Mini Kit (Qiagen). The culture medium was aspirated completely followed by adding 350 µl buffer RLT (lysis buffe). Cell lysate was collected by scraping and mixed by vortex. The lysate was homogenized by passing 5 times through a 20G needle fitted to a syringe. One volume of 70% ethanol was added to homogenized lysate and mixed by pipetting. The mixture was transferred to an RNeasy mini spin column, and centrifuged for 15 s at 8,000 *g*. The column was washed by adding 700 µl Buffer RW and centrifuged for 15 s 8,000 *g*. Thereafter, the column was treated twice by adding 500 µl buffer RPE and centrifuged for 15 s at 8,000 *g* and for 2 min at maximum speed in a regular centrifuge. RNA was recovered by eluting the column with 50 µl RNase-free water.

2.2.7 Polymerase chain reaction

Reverse transcription

Purified total RNA (0.5-2 µg) and 1 µl oligo dT (0.5 µg/µl) were added to DEPC treated water to final volume of 13.5 µl. The mixture was incubated at 70^{o}C for 5 min and quickly chilled on ice. The reverse transcriptase buffer, reverse transcriptase and dNTPs were added to the mixture and the final volume was adjusted to 20 µl by

adding DEPC treated water. Reverse transcription was performed by incubating at 42^{o}C for 1 hr followed by inactivation of enzyme at 94^{o}C for 5 min.

Polymerase chain reaction

Reaction mixture was prepared by adding 5 µl 10x PCR buffer, 0.5 µl PFU (2.5 unit/µl), 1 µl dNTPs (10 mM), 2 µl RT product, and 1 µl each of forward and reverse primers. Final volume was adjusted to 50 µl. The PCR reaction was performed in PCR machine at the setting as follow:

Step 1, 94 ^{o}C 5 min

Step 2, 94 ^{o}C 30 s

Step 3, 55-60 ^{o}C 45 s

Step 4, 72 ^{o}C 1 min

Repeat from step 2 to step 3 for 25-30 cycles,

Step 5, 72 ^{o}C 4 min

Step 6, 4 ^{o}C

2.3 Transfection and cell selection

2.3.1 Transfection using SUPERFECT

On the day before transfection, 2–8 x 10^5 cells were seeded in 5 ml of appropriate growth medium in 60-mm cell culture dishes. The cell number seeded should produce 40–80 % confluence on the day of transfection. The cells were then incubated at 37°C and 5% CO_2 in an incubator overnight. Five µg of plasmid DNA dissolved in TE, pH 7.4 (minimum DNA concentration: 0.1 µg/µl) was diluted with serum-free cell growth medium containing no antibiotics to a total volume of 150 µl. The solution was mixed

and spinned down for a few seconds to remove drops from the top of the tube. SuperFect transfection reagent (20 µl) (Qiagen) was added to the DNA solution, and mixed by pipetting 5 times or by vortexing for 10 s. The samples were incubated for 5–10 min at room temperature (20–25°C) to allow complex formation. While complex formation was taking place, the culture medium in the dishes containing cells was aspirated and cells were washed once with 4 ml PBS. One ml of cell growth medium (containing serum and antibiotics) was added to the reaction tube containing the transfection complexes and mixed by pipetting twice. The whole transfection medium was immediately transferred to the cells in the wells. Cells were then incubated with the complexes for 2–3 hrs at 37°C and 5% CO_2. Afterwards, transfection medium was aspirated and cells were washed 3–4 times with 4 ml of PBS. Thereafter, fresh cell growth medium (containing serum and antibiotics) was added, and cells were incubated for 24–48 hrs. Then cells were passaged at 1:10 to 1:15 splitting in the appropriate selecting medium, and cultured until clones appeared.

2.3.2 Transfection using FUGENE6

Cells were seeded in 6-well plates at density of 1-3 x 10^5 cells before the day of transfection, and incubated at 37°C and 5% CO_2 overnight. The transfection reagent:DNA complex was prepared by mixing 3 µl FuGENE6 reagent (Roche Diagnostics) and 1 µg plasmid DNA in serum-free medium sequentially, followed by incubation for a minimum 15 min at room temperature. The complex mixture was drop-wisely added to cells without changing medium, and cells were continuingly cultured in an incubator for 2 days. Subsequently, cells were passaged into selecting medium and cultured until clones appeared.

2.3.3 Cell selection for stable expression of transgenes

Transfected cells were continuing to culture in medium containing drugs (50 μg/ml hygromycin for Rac1 mutant transfected or 50 μg/ml geneticin for syncollin transfected cells, respectively) for a selection period of 4 weeks until individual clones were visible in culture dishes. The clones were then subcultured and used as polyclonal cell population for further experiments. These transfected polyclonal cells were verified by immunofluroscence staining and confocal microscopy to confirm the expression of transgenes in these cells (Fig. 3.2B demonstrated Rac1 mutant expression; Fig 3.19 revealed syncollin constructs expression). After selection, all transfected cells were cultured in medium containing 25 μg/ml drugs to maintain the cells stably expressing transgenes.

The cells used for experiments of intracellular co-localization of syncollin were performed by transient transfection for two days and directly for experiments without drug selection.

2.4 Subcellular fractionation

2.4.1 Buffers

Buffer	**Composition**
Homogenization buffer	20 mM Tris-HCl, pH 7.4; 0.5 mM EDTA; 0.5 mM EGTA; 0.25 M sucrose; 1 mM DTT; 10 μg/ml leupeptin, 4 μg/ml aprotinin; 2 μg/ml pepstatin; 100 μM PMSF
Medium A for plasma membrane	10 mM Tris-HCl, pH 7.4; 1mM MgCl; 0.25 M sucrose
Medium B for plasma membrane	10 mM Tris-HCl, pH 7.4; 1mM MgCl; 2.0 M

	sucrose
HEPES-buffered saline (HBS)	50 mM HEPES, pH 7.6; 100 mM NaCl; 1 mM EDTA; 1 mM DTT; 1 mM PMSF; 1 μg/ml pepstatin

2.4.2 Isolation of the plasma membrane

Plasma membrane isolation was modified from Graham's method (Graham, 1997). INS-1 cells (4.5 x 10^7) were harvested by scraping after twice washes with PBS. Thereafter, the cells were centrifuged at 800 rpm for 5 min and the pellet was resuspended in 1-2 ml homogenization buffer. The cells were homogenized by using a 27G needle with a syringe for 10 strokes. The homogenate was centrifuged at 250 *g* for 5 min. The supernatant was transferred to new tubes and centrifuged at 1,500 x g for 10 min. The pellet was resuspended in 1 ml medium A and mixed well with 2 volumes of medium B. The suspension was transferred to a 5-ml tube for a Beckman SW70 swing-bucket rotor, and filled by overlaying with 0.5 ml of medium A, followed by centrifuging at 113,000 *g* for 1 hr. The plasma membrane sheet at the interface was collected and diluted with 1 ml homogenization buffer and harvested by centrifugation at 3,000 *g* for 10 min. The pellets were resuspended in HBS and protein concentrations were quantified using Bio-Rad assay kit.

2.4.3 Subcellular fractionation of organelles

Subcellular fractions of INS-1 cells were separated as previously described (Li et al., 1996). All procedures were performed on ice. About 4.5 x 10^7 cells grown in 15-cm dishes were washed twice with cold PBS and scraped in 1 ml homogenization buffer. The cells were then disrupted by 10 strokes through a 27G needle. The cell homogenates were centrifuged at 900 *g* for 10 min to remove the nuclei and unbroken

cells. Mitochondria-enriched, secretory-granule-enriched and microsomal fractions were isolated by centrifuging post-nuclear supernatants at 5,500 *g* for 15 min (in Eppendorf 5417R), 25,000 *g* for 20 min and 100,000 *g* for 60 min (in Beckman L-80), respectively. For the isolation of total membrane and soluble fractions, the post-nuclear supernatants were centrifuged at 100,000 *g* for 60 min. All membrane fractions were resuspended in HBS. Protein concentrations in subcellular fractions were measured by Bio-Rad assay kit.

2.5 Protein analysis

2.5.1 Buffers for protein analysis

Buffer	Description
Lysis buffer	100 mM Tris-HCl, pH 8.0; 75 mM NaCl; 2 mM EDTA; 10 % glycerol; 1 μg/ml aproptinin; 1 μg/ml leupeptin; 0.5 % Triton X-100; 0.1 mM PMSF
Loading buffer (2x)	100 mM Tris-HCl, pH 6.8; 200 mM DTT; 20 % glycerol; 4 % SDS; 0.2 % bromophenol blue
SDS/PAGE buffer	0.1 mM sodium phosphate, pH 7.2; 0.1 % SDS
Blotting buffer	20% methanol; 24 mM Tris base; 194 mM glycine
Tris-buffered saline (TBST)	20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.2 % Tween-20;
Blocking buffer	TBST with 5 % non-fat milk powder

2.5.2 Antibodies

Antibody	Dilution	Supplier
Anti-c-Myc, monoclonal	1:200	Roche Diagnostics
Anti-Rac, monoclonal	1:1000	BD Bioscience
Anti-PIP5K-Iα, polyclonal	1:1000	Santa Cruz
Anti-Syncollin antiserum	1:40	Customer-ordered from GeneMed Synthesis, Inc., USA

2.5.3 Sample preparation

Cells cultured in dishes or multi-well plates were washed with cold PBS, scraped in a homogenizing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM

PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin) by cell-lifers, and sonicated on ice 3 times (10 s each). The samples were stored at -70^{o}C if not immediately used for Western-blotting.

2.5.4 SDS/Polyacrylamide gel electrophoresis (PAGE)

The 15% separating gel was prepared by mixing 6.7 ml monomer solution (30% Acrylamide/N,N'-methylenbisacrylamide solution), 5 ml 4X running gel buffer (1.5 M Tris-HCl, pH 8.8), 100 μl 10% SDS, 8.0 ml deionised distilled water, 100 μl 10% ammonium persulfate and 6.7 μl tetramethylethylenediamine (TEMED) in a total volume of 20 ml. The solution was well mixed and allowed to polymerize for at least 1 hr. Stacking gel was prepared by mixing 0.59 ml monomer solution (30% Acrylamide/N,N'-methylenebisacrylamide solutions), 1.11 ml 4X stacking gel buffer (0.5M Tris-HCl, pH 6.8), 44 μl 10% SDS, 2.71 ml deionised distilled water, 22.3 μl 10% ammonium persulfate and 2.3 μl TEMED. The mixture was overlaid on separating gel and allowed to polymerize for at least 1 hr. Proteins samples were loaded on the gel after mixing with loading buffer and heated at 95^{o}C for 5 min, followed by electrophoresis (20mA per gel) for 1-2 hrs.

2.5.5 Western blotting

Proteins separated in SDS/PAGE were transferred to a nitrocellulose membrane in blotting buffer by constant voltage (90V) for 2 hrs. Blotting membrane was incubated in blocking buffer for 1 hr at room temperature, followed by three washes with TBST. Thereafter, the membrane was incubated with primary antibody diluted in blocking buffer for 1 hr at room temperature or overnight at 4°C in gentle rotation. Following three washes with TBST, the membrane was incubated in diluted HRP-conjugated second antibody for 1 hr at room temperature with gentle rotation. After three washes,

the membrane was immersed in freshly-prepared enhanced chemiluminescence (ECL) substrate solution for 5-10 min. Image exposure was performed by using X-ray film or ChemiDoc Image capturer (Bio-Rad). Density quantification of bands was determined by the software Quantity One (Bio-Rad).

2.6 Measurement of Rac1 GTPase activity

Rac1 activity was determined by a pull-down assay using GST-PAK-CD (CD denotes to CRIB domain) as a probe that solely binds to active Rac1-GTP and Cdc42-GTP (Malliri et al., 2002). INS-1 cells (~1 x 10^7) were incubated with 2.8 mM or 15 mM glucose for 30 min. The cells were then washed with ice-cold PBS and incubated for 5 min on ice in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 30mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). Cell lysate were harvested and centrifuged for 5 min at 21,000 *g* at 4°C. Aliquots (100 μl) were taken from the supernatant for detecting total Rac1 amount. The remaining supernatants were incubated with 20 μg GST-PAK-CD fusion peptide (a generous gift from Dr. Edward Manser, Institute of Molecular and Cell Biology, Singapore) and 25 μl 50% glutathione-Sepharose 4B beads (Amersham, UK) for 60 min at 4 °C with gentle agitation. The mixture was centrifuged at 500 *g* for 5 min to sediment the matrix, followed by 3 washes with cold lysis buffer. The proteins were eluted by adding Laemmli sample buffer and boiled for 5 min. The eluted samples, including those supernatant aliquots, were subjected to SDS-PAGE and Western blotting as described above.

2.7 Immunofluorescence staining

2.7.1 Commonly used solutions

Solution	**Description**
Phosphate-buffered saline (PBS)	137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4; pH 7.4
Fix solution	3.7 % paraformaldehyde in PBS
Permeabilization solution	0.2 % Triton X-100 in PBS
Blocking solution	10 % fetal bovine serum in PBS

2.7.2 Antibodies

Antibody	**Dilution**	**Supplier**
Anti-insulin, monoclonal	1:1000	Sigma
Anti-Rac, monoclonal	1:20	BD Bioscience
FITC conjugated anti-Rac, monoclonal	1:20	BD Bioscience
Anti-c-Myc, polyclonal	1:20	Santa Cruz
Anti-c-Myc, monoclonal	1:20	Roche Diagnostic
Anti-calnexin, polyclonal	1:20	Santa Cruz
Anti-Mn superoxide dismutase, polyclonal	1:500	StressGen, Victoria, Canada
Rabbit anti-α-mannosidase II antiserum	1:1000	Dr. Kelley Moremer, University of Georgia, USA
TRITIC conjugated anti-mouse IgG, monoclonal	1:100	Sigma
FITC conjugated anti-rabbit IgG, polyclonal	1:20	Sigma
FITC conjugated anti-rabbit IgG, polyclonal	1:10	DAKO

2.7.3 Immunofluorescence microscopy

INS-1 cells cultured on coverslips were washed twice in PBS (pH 7.4) and fixed for 10 min at room temperature in 3.7% paraformaldehyde in PBS. Following 2 washes in PBS, the cells were permeabilized by 10-min incubation in PBS containing 0.2% Triton X-100. After 30 min blocking in PBS containing 10% fetal bovine serum, the cells were incubated in solution containing primary antibodies for 1 hour at room temperature. The coverslips were washed 6 times in PBS, and then incubated for 1 hr with TRITC/FITC-conjugated secondary antibodies (1:100 dilution) (Sigma, USA). Thereafter, coverslips were washed again 6 times in PBS and mounted on glass slides with fluorescence mounting medium (Dako, Carpinteria CA, USA). Samples were examined under laser confocal microscopy (Olympus IX 70 Fluoroview300 or Zeiss LSM510).

2.7.4 Rhodamine-phalloidin staining of filament actin

F-actin staining by phalloidin toxin was conducted as described previously (Li et al., 1994). INS-1 cells were seeded on glass coverslips and cultured for 2 days. After 2 washes with PBS (pH 7.4), cells on coverslips were fixed with 3.7% paraformaldehyde in PBS for 10 min. Cells were washed twice again and then incubated with 330 nM rhodamine-phalloidin (Molecular Probes, USA) and 300 ng/ml of lysophosphatidylcholine (Sigma, USA) for 20 min at room temperature. Subsequently, coverslips were washed twice with cold PBS and mounted on glass slides. The slides were examined by laser confocal microscopy (Olympus IX 70 Fluoroview300).

2.8 Insulin secretion assay

Insulin secretion was determined as described previously (Li et al., 1993). INS-1 cells were seeded in 24-well plates and cultured for 3-4 days in RPMI-1640 medium. For

secretion experiments, the cells were washed twice with fresh Krebs-Ringer-Hepes buffer (KRB) (containing in mM: 129 NaCl, 4.8 KCl, 1 $CaCl_2$, 1.2 $MgSO_4Cl_2$, 5 mM $NaHCO_3$, 1.2 KH_2PO_4, 2.8 glucose, and 10 Hepes, pH 7.4). After the cells were preincubated for 30 min in 0.5 ml KRB at 37°C, the buffer was replaced by 0.5 ml KRB containing secretagogues and the cells were incubated for the specified periods. The supernatants were removed for measurements of secreted insulin and the attached cells were extracted by acid-ethanol for determination of insulin content. Insulin was assayed by a radioimmunoassay kit (Linco Research, St. Charles, MO, USA). The rates of insulin secretion were expressed as percentages of insulin content to correct the possible influence of variations in the cell number between wells.

2.9 Measurement of cytosolic free calcium

Cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) were determined as described previously (Li et al., 2000). INS-1 cells (about 20 x 10^6) in flasks were trypsinized according to standard procedures. After washing, cells were resuspended in 50 ml RPMI-1640 medium supplemented with 1% new born calf serum and 20 mM Hepes, and kept in a spinner for 3 hrs at 37°C. Then the cells were washed once and resuspended in 20 ml spinner medium supplemented with 10% fetal calf serum, and left for 10 min at 37°C. Subsequently, the cells were loaded with a fluorescent Ca^{2+} probe by incubation with 1 μM Fura-2/AM for 30 min at 37°C. After washing, cells were aliquoted and about 2 × 10^6 cells in 2 ml of KRB were transferred to a glass cuvette. Stimuli were added to the cuvette after equilibration of cells at 37° for ~10 min. Fluorescence was recorded by a spectrofluorometer (Perkin Elmer LS-50B), with excitation and emission wavelengths of 340 and 505 nm, respectively. Unless specified, the contaminating fluorescence from extracellular Fura-2 was assessed by adding 50 μM Mn^{2+} (which was then

chelated by adding 100 μM diethylenetriaminepentaacetic acid) and was subtracted accordingly for each trace. Calibration of fluorescent signals into $[Ca^{2+}]_i$ values was carried out by a formula described previously (Li et al., 2000).

2.10 Measurement of membrane potential

Membrane potential of INS-1 cells was monitored using a voltage-sensitive fluorescent probe, bisoxonol, as described previously (Li et al., 2000). The anionic dye binds to the plasma membrane; its fluorescence is increased when cells are depolarized and decreased when cells are hyperpolarized. About 2×10^6 cells in KRB buffer were placed in a cuvette. Bisoxonol (final 100 nM) was added and cells were left at 37°C for about 15 min for equilibration. Fluorescence was recorded with excitation and emission wavelengths of 540 and 580 nm, respectively. For comparison and statistical analysis, the bisoxonol fluorescent signals were normalized by expression of results as the percentage of a near-maximal depolarization achieved by 40 mM KCl in each trace.

2.11 Assessment of nutrient metabolism by MTS test

Nutrient metabolism of the cells was monitored using an MTS assay kit (CellTiter 96^{tm}) developed by Promega. INS-1 cells were seeded onto 96-well plates and cultured in the presence of test agents for various periods. A mixture of MTS and PMS (phenazine methosulfate; an electron coupling reagent) (final concentrations 333 and 25 μg/mL, respectively) was added, and cells were incubated for 30 min at 37°. The reaction was stopped by the addition of 10% SDS if samples were not subjected immediately to the determination of absorbance. Formazan formed from reduction of MTS (Cory et al., 1991) was quantitated by measurement of absorbance of the medium

at 490 nm using a microplate reader. All data have been corrected for background signals.

2.12 Statistical analysis

Data were expressed as mean ± SEM and statistically analyzed by two-tail paired or unpaired *t*-test depending on individual experiments.

Chapter 3

Results

Part I: The role of Rac1 in glucose and forskolin stimulated insulin secretion in insulin-secreting β (INS-1) cells

3.1 Background

The Rho family of small GTP-binding proteins contains more than 10 members in mammals including Rho, Rac and CDC42 that are most well studied (Hall, 1998; Ridley, 1996). The Rho proteins are primarily involved in the regulation of actin cytoskeleton organization and are important for cell adhesion, migration, phagocytosis, cytokinesis and other morphological changes such as formation of ruffles, lamellipodial and filopodial extensions (Billadeau et al., 1998; Gasman et al., 1998; Hall, 1998; Hong-Geller and Cerione, 2000; Tatsumoto et al., 1999). In addition, there is evidence for the implication of Rho proteins and actin filaments in regulated exocytosis, although their actions seem quite complex (Aunis and Bader, 1988; Hall, 1998). The actin network beneath the plasma membrane was transiently depolymerized during exocytosis in mast cells and chromaffin cells (Koffer et al., 1990; Rose et al., 2001). Thus it appeared that the cortical actin cytoskeleton web acts as a barrier to hamper the access of secretory granules to the plasma membrane and a reorganization of F-actin in this region occurs during the late steps of exocytosis. In pancreatic β-cells, disrupting F-actin by cytochalasin B facilitated insulin release (Jijakli et al., 2002), in particular during the first phase (Li et al., 1994). On the other hand, maintenance of F-actin structure is also required for secretory process. For instance, an increase of F-actin was observed when pancreatic β cells were stimulated

by glucose (Swanston-Flatt et al., 1980), and cholecystokinin-induced exocytosis in permeabilized pancreatic acinar cells was inhibited when the highly concentrated monomeric actin binding protein β-thymosin was introduced (Muallem et al., 1995). Moreover, disruption of F-actin filaments by using *Clostridium botulinum* C2 toxin preferably reduced the second phase of insulin secretion from islet β-cells (Li et al., 1994). Thus, the basic substructure of actin cytoskeleton may also be essential for the normal recruitment of secretory granules to the plasma membrane. In addition, actin network may provide contractile forces that expel the granule contents (Valentijn et al., 1999), since earlier studies had demonstrated that inhibition of myosin light chain kinase reduced insulin secretion from β-cells (Li et al., 1994).

It was uncertain that Rho proteins participated in the modulation of exocytosis through regulating cytoskeleton reorganization or by other mechanism. Rho A is possibly involved in exocytosis in chromaffin cells by regulating phosphatidylinositol-4-kinase (Gasman et al., 1998). However, inactivation of Rho by *botulinum toxin* C3 did not affect stimulated insulin release (Kowluru et al., 1997b). It appeared that Rac proteins are particularly interesting for this context as evidenced in several secretion systems, including mast cells, chromaffin cells and neuronal cells (Brown et al., 1998; Doussau et al., 2000; Gasman et al., 1999; Komuro et al., 1996; Price et al., 1995). At least three Rac isoforms have been identified in mammals. Rac1 is ubiquitously expressed (Haataja et al., 1997; Moll et al., 1991), while Rac2 is a hematopoietic-specific and Rac3 is highly expressed in brain (Haataja et al., 1997; Shirsat et al., 1990). Rac3 shares 92% and 89% amino acid identity with human Rac1 and Rac2, respectively (Haataja et al., 1997). Application of Rac mutants in permeabilized cells is a direct approach to observe its instant effect on secretion. It was found that constitutively active Rac enhanced regulated secretion whereas dominant-inhibitory Rac exhibited

opposite effects in permeabilized mast cells (Brown et al., 1998; Price et al., 1995). Studies using clostridial toxins to specifically inactivate certain members of Rho proteins in pancreatic β cells also implied that Rac and Cdc42 rather than Rho may be the candidate regulators implicated in stimulated insulin secretion (Kowluru et al., 1997b).

The mechanism of possible role of Rac GTPase, such as its response to glucose stimulation, in regulated insulin secretion was unclear. Therefore, in the present study dominant inhibitory or constitutively active Rac1 mutants were expressed in insulin-secreting INS-1 β cells to address this question.

3.2 Differential distribution of expressed Rac1 mutants from endogenous Rac1

In control INS-1 cells (transfected with empty vectors), the endogenous Rac1 was mainly present in cytosol even though trace amounts were detected in the fractions enriched with mitochondria and secretory granules, as assessed by Western-blotting (Fig. 3.1***A***). The examination by immunofluorescence staining indicated that native Rac1 was distributed in whole cytoplasm (Fig. 3.1***B)***.

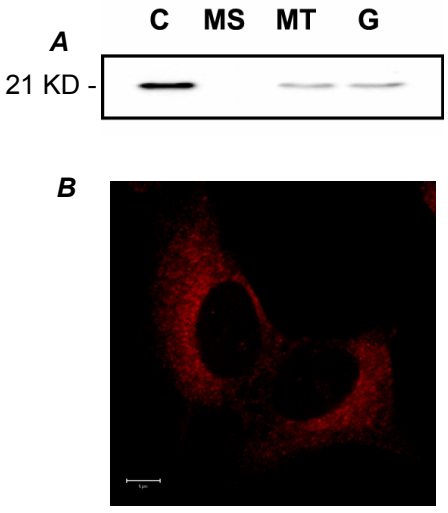

Fig. 3.1. Distribution of endogenous Rac1 in INS-1 cells. (***A***) Immunoblotting detection of Rac1 in subcellular fractions. Cells were homogenized and separated by differential centrifugation to obtain mitochondria-rich (MT), insulin granule-rich (G), microsomal (MS) and cytosol (C) fractions. Equal amount (20 μg) of proteins from each fraction was separated by SDS-PAGE and probed by an anti-Rac1 monoclonal antibody. Data are the representative of at least 4 experiments with identical results. (***B***) Immunofluorescence staining of Rac1 in INS-1 cells was probed by anti-Rac1 monoclonal antibody and TRITC conjugated anti-mouse IgG. Bar=5 μM

The cells expressing Rac1-mutants used for the experiments were polyclonal cells derived from stable trnasfection through selection with antibiotic hygromycine (Roche Diagnostic) and verified by using Western blotting and immunifluorescence staining. A majority of the transfected cells were positive for the mutant proteins (Fig. 3.2B).

An antibody recognizing exclusively Myc-tagged proteins was employed for immunofluorescence staining of Rac1 mutants that contain the tag. No signal could be detected in control cells in this way (Fig.3.2). Although the staining in dominant inhibitory N17Rac1 transfected cells was observed primarily in the soluble compartment, considerable staining dots were also detected around the cell periphery (Fig. 3.3, left panel). The constitutively active V12Rac1 was more evenly distributed in the cells (Fig. 3.3, right panel). Western blot analysis of the subcellular fractionations yielded additional details of the localization of Rac1 mutants in transfected cells (Fig. 3.4). In contrast to the endogenous Rac1 that was mainly localized in the cytosol, N17Rac1 was primarily detected in the fractions enriched with mitochondria or insulin granules (Fig. 3.4, lane 1). However, V12Rac1 was distributed in all fractions including the microsomal and cytosolic fractions (Fig. 3.4, lane 3). These results reveal differential distribution patterns for the endogenous and mutated Rac forms among various subcellular fractions.

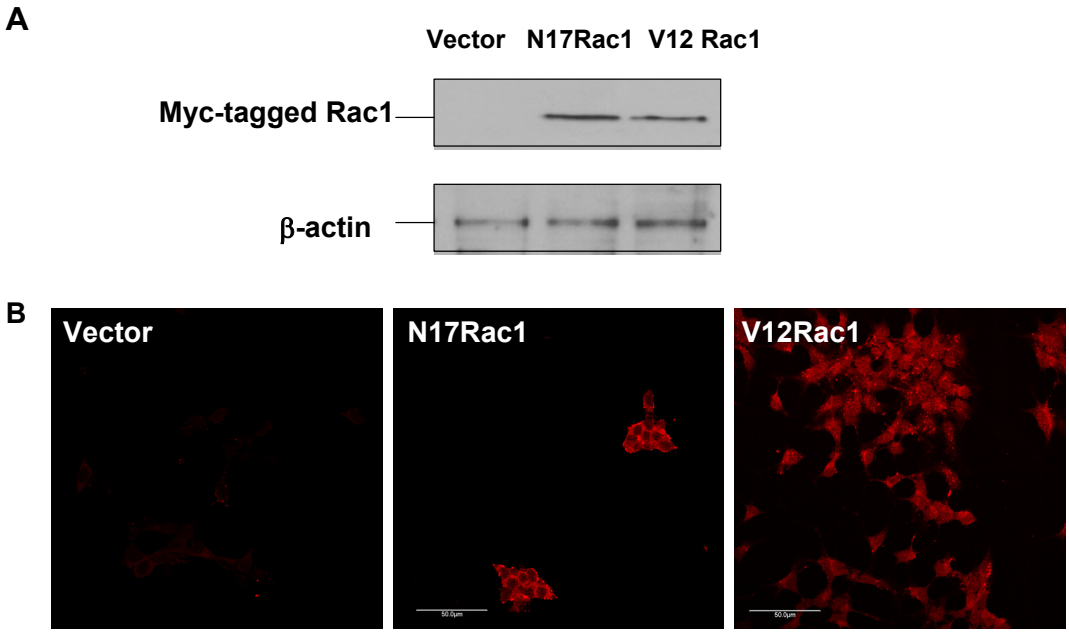

Fig. 3.2. Identification of INS-1 cells stably expressing Rac1 mutants after hygromycin selection. A. Western blotting assay by using anti-c-Myc antibody demonstrated that the transfected cells express Myc-tagged Rac1 mutants. β-actin was used as internal control**. B.** Immunofluorscence staining by using anti-c-Myc antibody and TRITC conjugated anti-mouse IgG verified polyclonal cell population after hygromycin selection in Rac1 mutants.stably transfected INS-1 cells. Images were detected by Olympus IX70 Fluoroview 300 laser confocal microscopy. Bar=50μm.

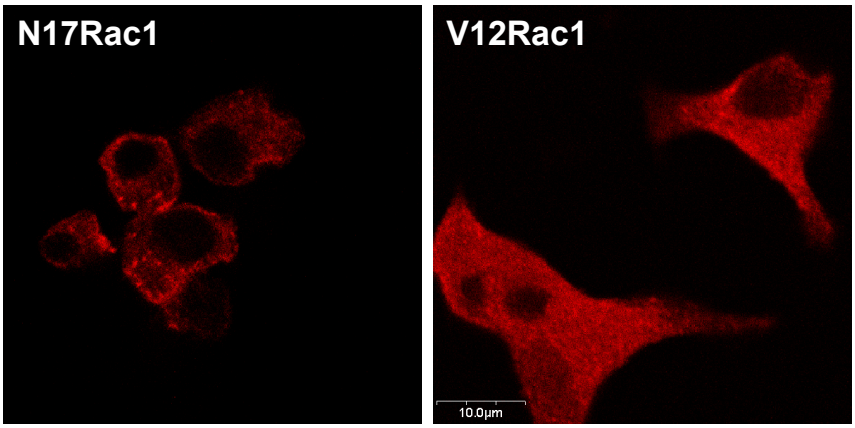

Fig. 3.3. Intracellular localization of stably expressed Rac1 mutants in Ins-1 cells. Stably expression of Rac1 mutants, N17Rac1 and V12 Rac1, in INS-1 cells was examined by immunofluorescence staining with anti-c-Myc monoclonal antibody and TRITC-conjugated anti-mouse IgG. Images were detected by Olympus IX70 Fluoroview 300 laser confocal microscopy. Bar=10μm.

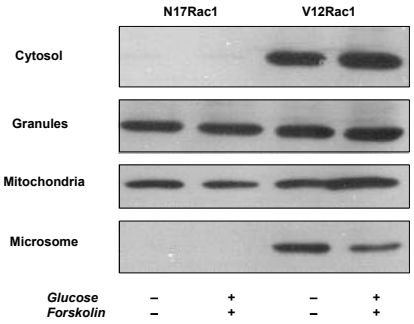

Fig. 3.4. Distribution of Rac1 mutants in INS-1 cells. Cells transfected by Rac1 mutants were incubated for 30 min in the presence or absence of 15 mM glucose plus 1 μM forskolin. The cells were then homogenized and subcellular fractions were isolated by differential centrifugation. Equal amount (20 μg) of proteins in each fraction was separated by SDS-PAGE and probed by immunoblotting with anti-c-Myc antibody. Data are the representative of at least 5 observations

3.3 Glucose specifically stimulates translocation of Rac1 from cytosol to membranes in control, but not in cells expressing the mutated Rac1

When control INS-1 cells were stimulated by 15 mM glucose for 30 min, there was an increase of Rac1 in the membrane fraction with a concomitant reduction in cytosol (Fig. 3.5B). Relative abundance of Rac1, expressed as its membrane to cytosolic ratio, was significantly increased (1.57 fold; from 0.19 ± 0.02 to 0.49 ± 0.04) following exposure to glucose (Fig. 3.5A). The cAMP-elevating agent forskolin (1 μM) did not significantly affect the degree of Rac1 translocation elicited by glucose. Time course of glucose-induced Rac1 translocation revealed that such an effect reached significance at 15 min (Fig. 3.5C). Further studies revealed that glucose treatment is able to specifically facilitate translocation of Rac1 into the plasmalemma fraction from the cytosol (Fig. 3.6).

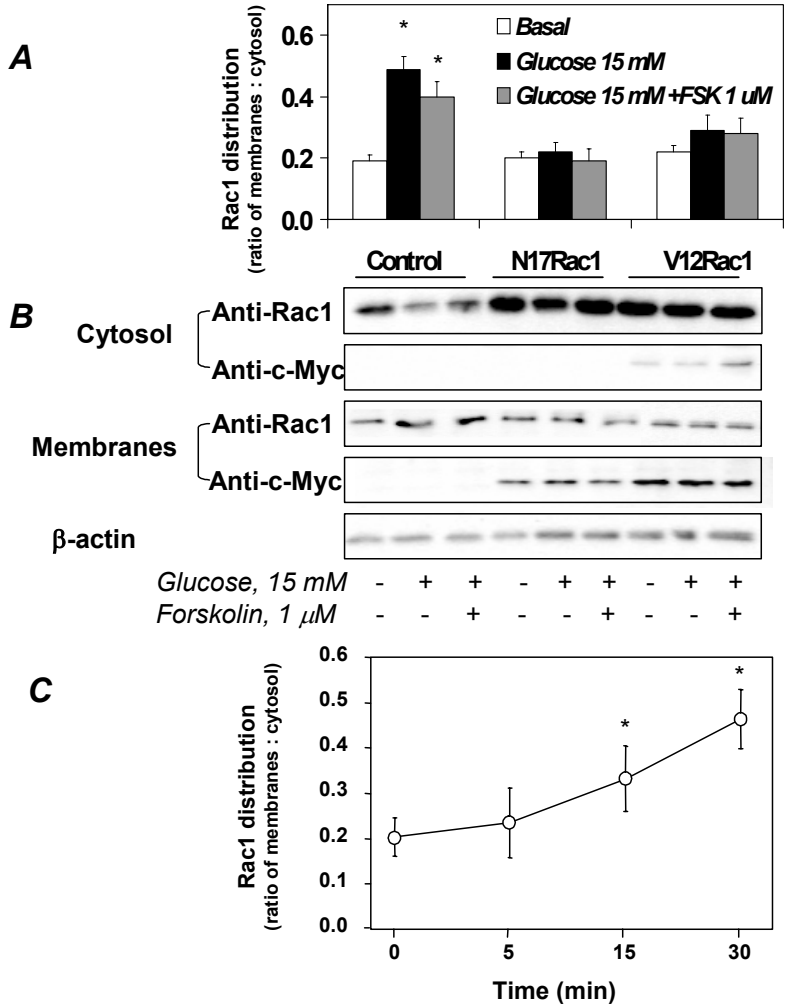

Fig. 3.5. Glucose-induced translocation of Rac1 in INS-1 cells. (***A*** and ***B***) Following stimulation of cells with 15 mM glucose alone or 15 mM glucose plus 1 μM forskolin for 30 min, the total membrane fraction and cytosolic fraction were isolated (see Methods for additional details). The fractions from control and Rac1 mutant-transfected cells were first probed by immunoblotting using an anti-Rac1 monoclonal antibody recognizing both native and mutated Rac1. Thereafter, the membranes were striped and an anti-c-Myc monoclonal antibody was used to probe Rac1 mutant in transfected cells. β-actin was used as internal control. The data in (***A***) denote the total Rac1 translocation. (***C***) Time course of glucose-induced Rac1 translocation in control INS-1 cells. Data are the representative of at least 4 (for ***A*** and ***B***) and mean ± SEM of 4 (for ***C***) observations. * P<0.05 *vs*. control.

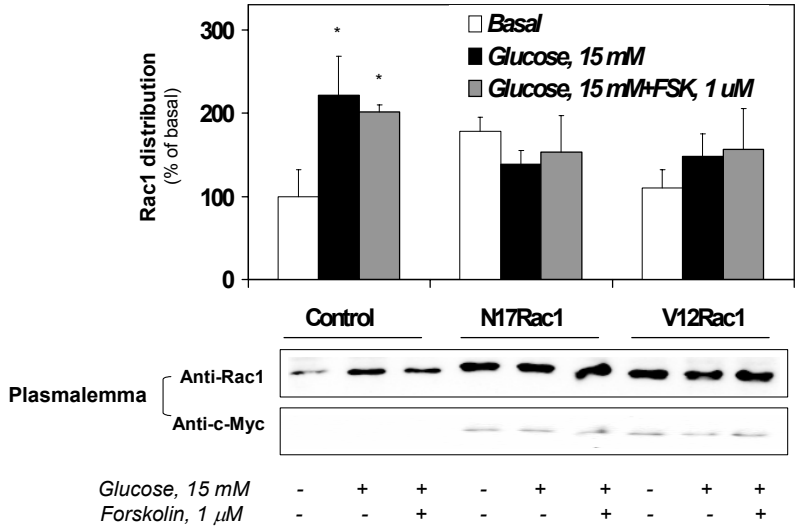

Fig. 3.6. Translocation of Rac1 to the plasmalemma by glucose. Following stimulation of cells by 15 mM glucose alone or plus 1 μM forskolin for 30 min, plasmalemma were prepared and probed by immunoblotting using an anti-Rac1 monoclonal antibody. Thereafter, the membranes were striped and an anti-c-Myc monoclonal antibody was used to probe Rac1 mutant. The bar graph denotes the total Rac1 in the plasmalemma. Data are the representative of at least 4 observations. * $P<0.05$ *vs*. control.

In contrast, stimulating cells with 15 mM glucose plus forskolin for 30 min did not alter Rac1 distribution between cytosol and membranes in either Rac1 mutant transfected cells (Fig. 3.4, lanes 2 and 4), when probed by an antibody that recognized solely the Myc-tagged Rac1 mutants. Interestingly, even though the basal levels of membrane-associated Rac1 were higher in transfected cells compared to those expressing the vector alone (e.g., by 32% and 62% in N17Rac1- and V12Rac1-transfected cells, respectively) when assessed by an antibody that recognized both endogenous Rac1 and transfected Rac1 mutants, further stimulation of these cells expressing the mutants by glucose failed to induce Rac1 translocation from cytosol to the membranes (Fig. 3.5), including plasmalemma (Fig. 3.6). With the same approach, no apparent difference could be observed in the total Rac1 (both endogenous and mutated) contents in cell homogenates between the two Rac1 mutant-transfected cells and control cells (Fig. 3.7), indicating that there was no apparent over-expression of Rac1 mutants in the stably transfected INS-1 cells. These data suggest that glucose is

able to facilitate translocation of Rac1 (an indication of Rac1 activation) only in control cells, but not in cells transfected with mutated Rac1.

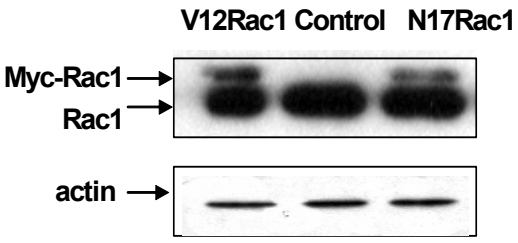

Fig. 3.7 Expression of Rac1 mutants does not affect the total Rac1 level in INS-1 cells. Immunoblotting assay for lysates (40 μg) of Rac1 mutant and empty vector transfected cells was performed by using anti-Rac1 antibody. Actin (42 Kd) was used as internal control. The Myc-tagged Rac1 has slightly larger molecular mass than that of the endogenous Rac1 becuase the c-Myc sequence used as a tag contains only 10 animo acids (EQKLISEEDL).

3.4 Glucose increases Rac1 activity as assessed by measuring active GTP-Rac1

Activation of Rac1 by glucose was confirmed by the direct detection of Rac1 GTPase activity. By using a fusion peptide (GST-PAK-CD) that is solely bound to the activated Rac1 (GTP-Rac1) as a probe (Malliri et al., 2002), GTP-bound Rac1 (i.e. elevation of Rac1 activity) was significantly increased by 15 mM glucose stimulation in control INS-1 cells over 30 min (Fig. 3.8). The Rac1 activity stimulated by high glucose was attenuated in dominant inhibitory Rac1 transfected cells (Fig. 3.8). A strong signal of GTP-bound Rac1 was observed in the cells expressing constitutively active Rac1 mutant and glucose stimulation was unable to further increase the activation (Fig. 3.8)

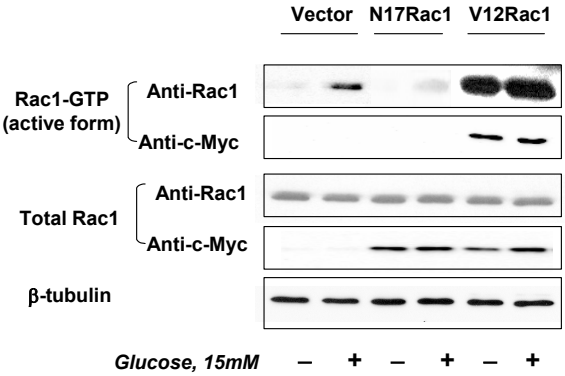

Fig. 3.8. Increase of activated Rac1 (Rac1-GTP) by glucose stimulation in INS-1 cells. A pull-down assay using GST-PAK-CD was utilized to detect the activated Rac1 in INS-1 cells after incubation with 2.8 or 15 mM glucose for 30 min (upper panel). Total Rac1 contents and Rac1 mutants in the lysate samples (20 μg proteins) were shown in the lower panel. Rac1 was probed by Western blotting as detailed in the Methods section. Data are the representative of 3 independent observations.

3.5 Dominant negative Rac1 causes marked morphological change in INS-1 cells

The control INS cells grew in monolayer on the culture surface with substantial lamellipodia (Fig. 3.9A). Remarkable changes in the morphology occurred in N17Rac1-transfected cells, which had less lamellipodia (a known morphological marker of Rac function (Hall, 1998) and tended to aggregate and stack (Fig. 3.9B). These cells rounded up and their ability to adhere to the substratum was also weaker since they were more easily detached by trypsinization. Besides, the size of these cells was significantly smaller (diameter of 9.9 vs. 12.6 nm of control cells) in suspensions. By contrast, INS-1 cells expressing the constitutively active V12Rac1 only revealed slight morphological changes, including more protrusions (Fig. 3.9C). In addition, the adhesion of these cells to substratum was relatively stronger (more resistant to trypsinization). These observations indicated that active Rac1 may be important for INS-1 cells to maintain normal morphology.

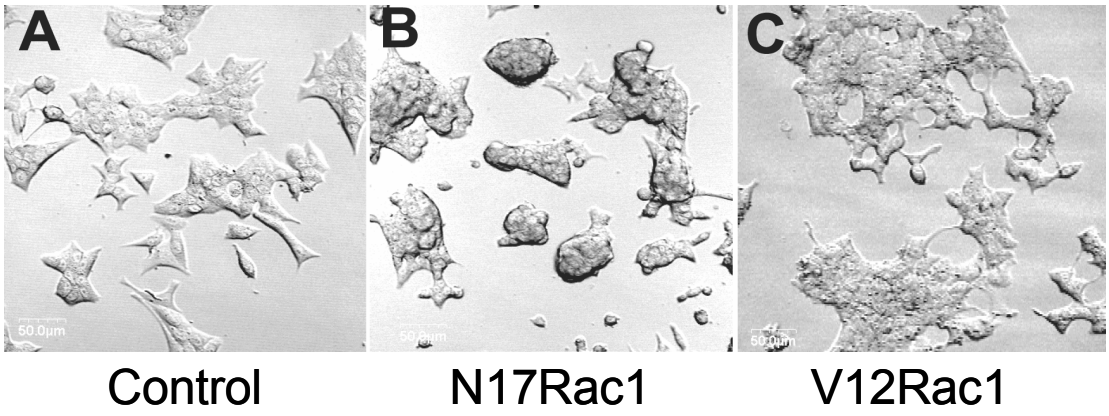

Fig. 3.9. Effects of expressing Rac1 mutants on INS-1 cell morphology. Cells were cultured in coverslip chambers. Living cell morphology was examined under differential interference contrast (DIC) by laser confocal microscopy. Images are the representative of at least 5 observations. Bar = 50 μm.

3.6 Expression of Rac1 mutants results in marked disruption of F-actin filaments in INS-1 cells

The observed morphological changes, as indicated above, might be due to the interference with cytoskeleton organization in the transfected cells. In support of such a formulation, the control INS-1 cells displayed strong F-actin distribution in cell periphery and also some filaments in the interior of cells, as assessed by rhodamine-phalloidin staining (Fig. 3.10, left). By contrast, F-actin staining in dominant inhibitory N17Rac1 transfected cells almost completely disappeared (Fig. 3.10, middle). It may be mentioned that even though some modest degree of reduction of F-actin staining was observed in constitutively-active V12Rac1 transfected cells, structure of F-actin filaments remained largely intact in these cells (Fig. 3.10, right). These findings suggested that the organization of F-actin filaments in INS-1 cells requires functional Rac1.

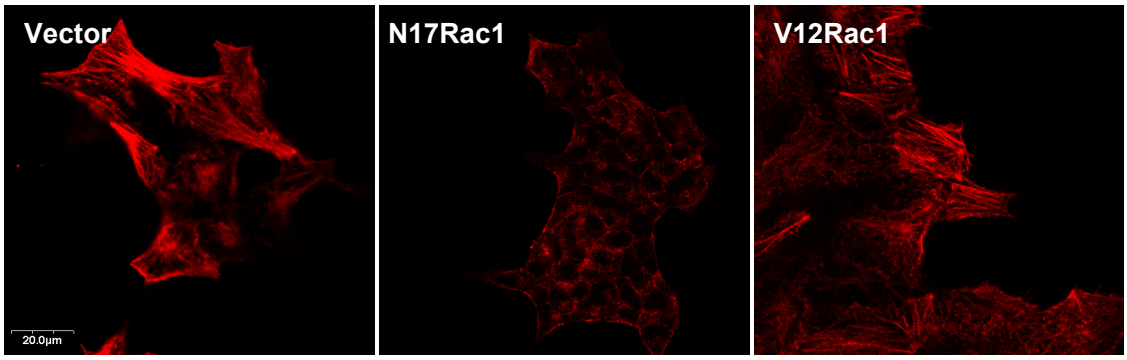

Fig. 3.10. F-actin organization in INS-1 cells expressing Rac1 mutants. Cells cultured on coverslips were fixed and stained by rhodamine-phalloidin which selectively binds to F-actin. The stained cells were examined by laser confocal microscopy. The images are the representative of 5 independent experiments. Bar = 20 µm.

3.7 Expressing dominant negative Rac1 inhibits glucose- and forskolin-stimulated insulin secretion in INS-1 cells

Stable expression of dominant inhibitory or constitutively-active Rac1 mutants did not alter either the insulin contents in INS-1 cells (see Table 3.1; p.65), or the basal insulin secretion (Fig. 3.11). Insulin secretion from control cells over 30 min was increased by 90% following exposure of these cells to 15 mM glucose. Simultaneous incubation with glucose (15 mM) and forskolin (1 µM) resulted in marked stimulation of insulin secretion (>290%) from these cells (Fig. 3.11). Expression of dominant inhibitory N17Rac1 significantly inhibited glucose-induced (>50%) and glucose plus forskolin-induced (60%) insulin secretion (Fig. 3.11). However, insulin release induced by glucose alone or glucose plus forskolin was not significantly affected in cells expressing constitutively-active V12Rac1. In addition, high potassium-stimulated secretion was moderately, but not significantly, reduced in cells transfected with either of the Rac1 mutants (Fig. 3.11). These data indicated that Rac1 might be involved in glucose and forskolin stimulated insulin secretion from INS-1 cells, and such

regulatory effects of Rac1 appear to take place at steps prior to initiation of exocytosis by Ca^{2+}, since insulin secretion elicited by a membrane-depolarizing concentration of KCl was unaffected in cells expressing the dominant negative form of Rac1.

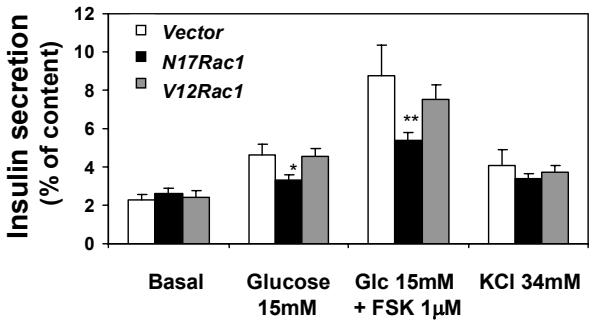

Fig. 3.11. Inhibition in glucose- and glucose plus forskolin-induced insulin secretion from INS-1 cells expressing dominant negative Rac1 mutant. Cells were cultured in 24-well plates. After stimulation of cells by secretagogues for 30 min, insulin secretion and insulin content were measured by RIA. The values are mean ± SEM of at least 5 independent experiments in triplicates. Glc, glucose; FSK; forskolin. * P<0.05 and ** P<0.01 *vs*. control.

Furthermore, stable expression of wild type Rac1 (Fig. 3.12A) also did not alter secretagogues-induced insulin release (Fig. 3.12B) and morphology of these cells remained unchanged, suggesting the specific N17Rac1 effects due to interference with Rac1. These data indicated that Rac1 may be involved in glucose and forskolin stimulated insulin secretion from INS-1 cells.

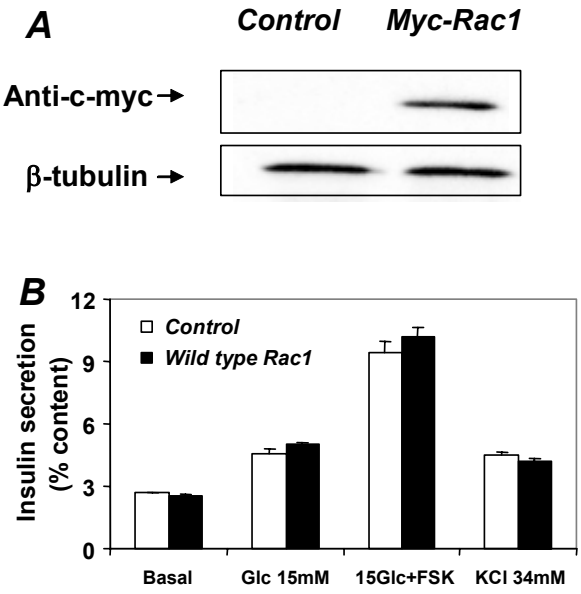

Fig. 3.12. No alteration of secretagogues-stimulated insulin release by expression of wild Rac1 in INS-1 cells. ***A,*** INS-1 cells were transfected by c-myc-taggered wild Rac1 or empty vectors (control), and stably selected by hygromycin. Expressed wild Rac1 was detected by Western blotting of cell homogenates (20 μg protein) using an antibody against c-myc. ***B,*** Cells cultured in 24-well plates were first preincubated for 30 min in KRB and then incubated with 2.8 mM glucose (basal) or specified secretagogues for 30 min. Insulin secretion and insulin content were measured by RIA. The values are mean ± SEM of 3 independent experiments in triplicates. Glc, glucose; FSK; 1 μM forskolin.

3.8 Expression of dominant negative Rac1 mutant leads to inhibition of the late phase of glucose plus forskolin-stimulated insulin secretion

It is well known that glucose evokes biphasic pattern of insulin secretion from islet β-cells and each of the two phases are felt to be controlled by different mechanisms (Bratanova-Tochkova et al., 2002; Henquin et al., 2002; Rorsman et al., 2000). To determine the possibly distinct roles of Rac1 in the two phases, insulin secretion from the dominant inhibitory Rac1 transfected cells was measured during two consecutive stimulating periods (15 min as early phase and followed by a 20 min incubation reflecting the late phase), an approach used previously by other peers to study the early and late phase insulin secretion responses to glucose in isolated islets (Nesher et al., 1989). The potentiating effect of forskolin on glucose-stimulated insulin secretion was greater (by 165%) in the late phase than the early phase (by 101%) in the control cells (Fig. 3.13). In cells expressing N17Rac1, the early phase of insulin release stimulated by glucose plus forskolin was significantly inhibited (-42%), albeit without significant effect on the release elicited by glucose alone (Fig. 3.13A). Furthermore, in cells expressing the dominant negative form of Rac, the degree of insulin secretion induced by either glucose alone or plus forskolin during the late phase was markedly inhibited by 63% and 49%, respectively (Fig. 3.13B). These results implicated that functional activation of Rac is essential for the late phase of insulin release, an effect that may be related to the Rac1 translocation (activation) by glucose during this period (cf. Fig. 3.5C).

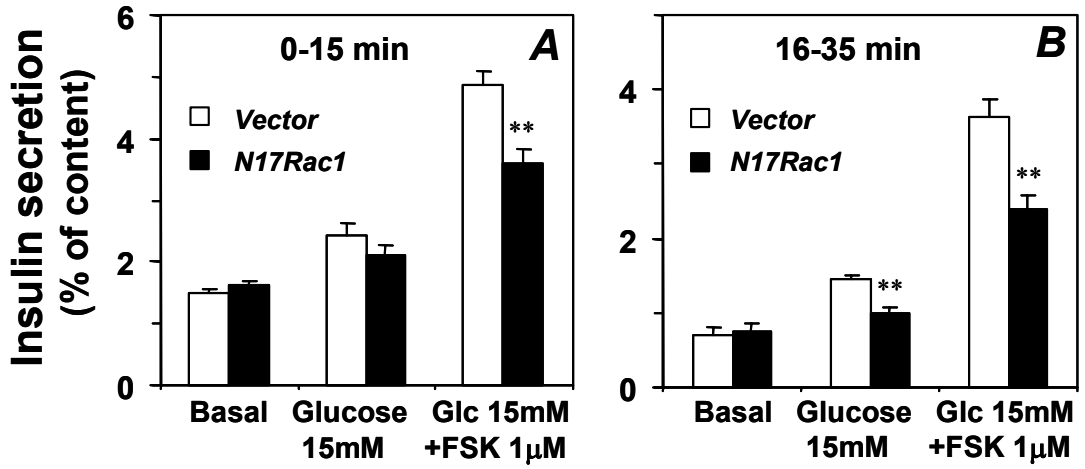

Fig. 3.13. Effects of dominant inhibitory Rac1 expression on the two static phases of insulin secretion induced by glucose and forskolin. Cells cultured in 24-well plates were stimulated by 15 mM glucose alone or plus 1 μM forskolin for 15 min incubation and the medium was removed for determination of the early secretion event. Afterwards, the cells were incubated with the fresh solution containing the same stimuli for another 20 min for assessment of the late phase secretion. The values are mean ± SEM of at least 3 independent experiments in triplicates. FSK; forskolin. **P<0.01 *vs*. control.

3.9 Glucose induces translocation of PIP5K-Iα from cytosol to membranes in control INS-1 cells, but not in cells expressing mutated Rac1

Based on the above observation of Rac1 translocation upon the stimulation of glucose, it was expected that Rac1 downstream effectors might be brought to the membranes where they interacts with Rac1. PIP5K-Iα, an isoenzyme of phosphatidylinositol-4-phosphate 5'-kinase, is an effector of Rac capable of directly interacting with Rac in a GTP-independent manner (Tolias et al., 2000). Its product, phosphatidylinositol-4,5-diphosphate (PIP_2) is also a potential regulator of actin cytoskeleton organization and may play a role in the process of priming for exocytosis (Hay et al., 1995). It is thus possible that PIP5K-Iα may mediate the effect of Rac1 on the regulated insulin secretion.

When control cells were stimulated by 15 mM glucose for 30 min, the membrane to cytosol distribution ratio of PIP5K-Iα was significantly increased (1.8 fold; from 3.65

± 0.13 to 6.55 ± 1.87) (Fig. 3.14). Addition of 1 μM forskolin further enhanced the effect (3.2 fold; from 3.65 ± 0.13 to 11.81 ± 2.44). However, these effects of glucose and forskolin on PIP5K-Iα translocation were not observed in the cells stably transfected with either dominant inhibitory Rac1N17 or constitutively active Rac1V12 (Fig. 3.14). The similar translocation pattern of PIP5K-Iα to that of Rac1 over glucose stimulation suggested a potential role of PIP5K-Iα in mediating the action of Rac1 on actin cytoskeleton reorganization and regulated insulin release.

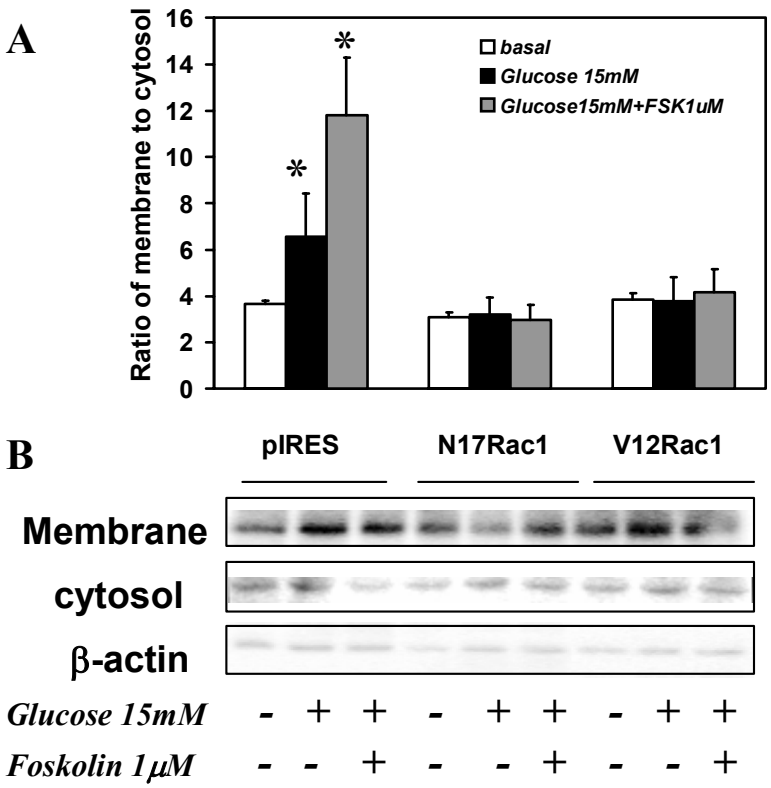

Fig. 3.14. Glucose-induced translocation of PIP5K-Iα in INS-1 cells. (***A*** and ***B***) Following stimulation of cells with 15 mM glucose alone or 15 mM glucose plus 1 μM forskolin for 30 min, the total membrane fraction and cytosolic fraction were isolated. The fractions from control and Rac1 mutant-transfected cells were probed by immunoblotting using an anti-PIP5KIα polyclonal antibody. Data are mean ± SEM (***A***) and the representative of at least 4 (***B***) observations. * P<0.05 *vs*. control.

3.10 Dominant inhibitory Rac1-mediated inhibition of insulin secretion does not appear to affect nutrient metabolism, membrane potential and $[Ca^{2+}]_i$ increases

To investigate the possible effects of dominant inhibitory Rac1 on the upstream signaling events in insulin secretion, nutrient metabolism, membrane potential and $[Ca^{2+}]_i$ increment were also examined upon stimulation of INS-1 cells. Incubation of control cells with 15 mM glucose for 30 min increased MTS reduction (reflecting metabolism) by about 3 folds over basal; an effect not significantly influenced by the expression of either Rac1 mutants (Table 3.1). In addition, there were no significant differences in either resting membrane potential or depolarization (induced by 15 mM glucose, 100 μM ATP, or submaximal KCl) between control and N17Rac1 transfected cells (Fig. 3.15, A-D and Table 3.1). Furthermore, $[Ca^{2+}]_i$ increments were also not affected by the expression of dominant inhibitory Rac1 when cells were stimulated by 15 mM glucose or 34 mM KCl (Fig. 3.15, E-H and Table 3.1). These findings suggested that functional activation of Rac1 may not be a prerequisite for the generation of proximal signals required for the exocytotic secretion of insulin in INS-1 cells elicited either by glucose or KCl.

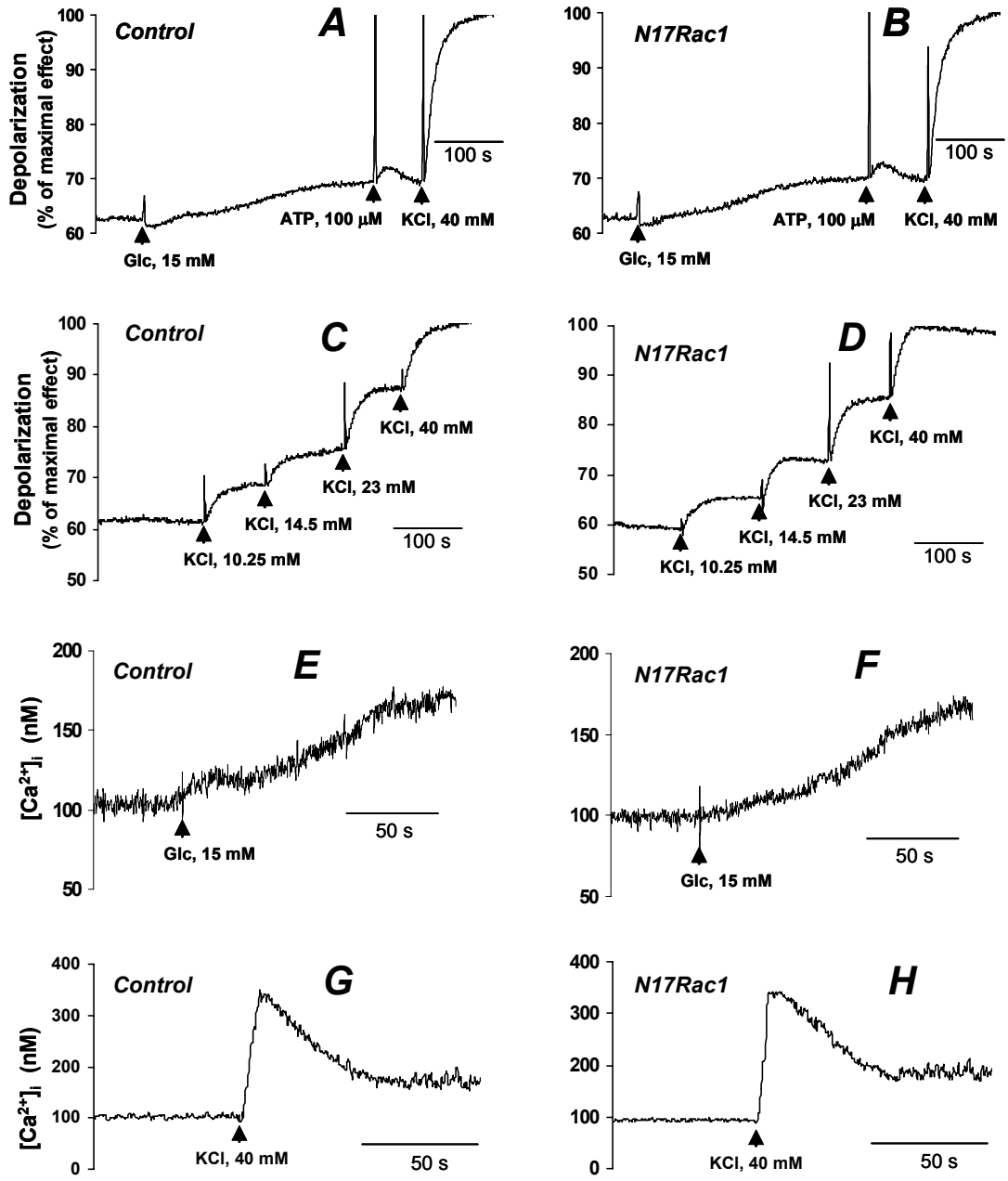

Fig. 3.15. Effects of expression of Rac1 mutants on membrane depolarization and $[Ca^{2+}]_i$ under various stimulating conditions. ***A-D***: Membrane potential of INS-1 cell suspensions was monitored with the voltage-sensitive fluorescent probe bisoxonol. Increase of fluorescence (upward) indicates depolarization. The results were expressed as percentage of the depolarizing signal achieved with a saturating concentration of KCl (40 mM) for each trace. The large, transient spikes are the artifacts caused following addition of test agents whereas the sustained changes reflect the true steady state values. ***E-H***: $[Ca^{2+}]_i$ in INS-1 cell suspensions was measured using a fluorescent probe (fura-2) as detailed in the Materials and Methods section. All traces are representatives of at least 3 observations in each case. The indicated values of KCl additions in the figure denote the final concentrations reached in the medium; basal K^+ concentration was 6 mM. Glc; glucose.

Table 3.1. No changes in insulin content, MTS test, membrane potential and $[Ca^{2+}]_i$ profile by transfection of Rac1 mutants in INS-1 cells

	Control	N17Rac1	V12Rac1
Insulin content (ng/ng DNA)	0.52 ± 0.07 (4)	0.50 ± 0.10 (4)	0.49 ± 0.06 (4)
Formazan production at glucose-free (arbitrary unit/10^6 cells)	0.45 ± 0.05 (4)	0.46 ± 0.06 (4)	0.48 ± 0.06 (4)
Formazan production by 15 mM glucose (arbitrary unit/10^6 cells)	1.15 ± 0.12 (4)	0.93 ± 0.10 (4)	1.09 ± 0.13 (4)
Rest membrane potential (% maximal effect)	62.9 ± 1.1 (6)	61.6 ± 2.1 (6)	64.8 ± 2.7 (6)
Depolarization by 15 mM glucose (% maximal effect)	69.5 ± 1.2 (6)	67.8 ± 1.9 (6)	71.7 ± 2.6 (6)
Depolarization by 100 μM ATP (% maximal effect)	72.3 ± 1.5 (6)	72.8 ± 1.8 (6)	73.5 ± 1.6 (6)
Depolarization by 23 mM KCl (% maximal effect)	86.4 ± 1.3 (6)	85.7 ± 1.9 (6)	88.5 ± 1.8 (6)
Basal $[Ca^{2+}]_i$ (nM)	108 ± 9 (3)	98 ± 7 (3)	N.D.
$[Ca^{2+}]_i$ increase by 23 mM glucose (nM)	178 ± 21 (3)	175 ± 13 (3)	N.D.
$[Ca^{2+}]_i$ increase by 40 mM KCl (nM)	309 ± 11 (3)	318 ± 21 (3)	N.D.

Formazan production from MTS test was measured over 30-min incubation. Maximal membrane depolarization was achieved with a saturating concentration of KCl (40 mM). The indicated conditions of KCl stimulation are the final concentrations of K^+ in the incubation medium. The numbers of experiments are indicated in the parentheses. N.D., not determined.

3.11 Stable expression of dominant negative Rac1 inhibits mastoparan-induced insulin secretion

To further confirm that Rac is an important regulatory protein in insulin secretion, mastoparan-stimulated insulin secretion was also studied in INS-1 cells stably expressing vector alone or the dominant inhibitory Rac1. Mastoparan is a potent activator of G-proteins, in particular for trimeric G proteins. The data showed in Fig. 3.16 demonstrated that mastoparan (30 μM for 45 min) markedly stimulated insulin secretion. Furthermore, a significant reduction (by ~30%) in mastoparan-induced insulin secretion was observed in INS-1 cells expressing N17Rac (Fig. 3.16). The results indicated that Rac represents one of the regulatory proteins involved in mastoparan-induced insulin secretion from β-cells.

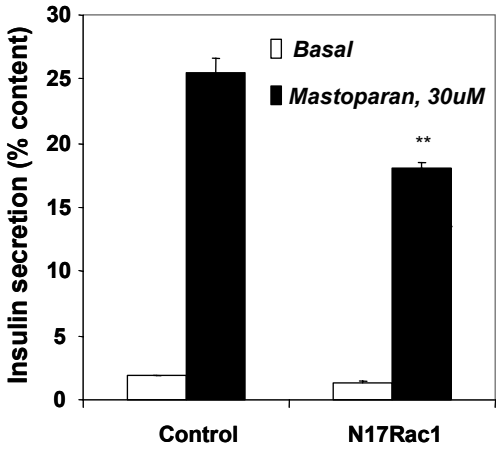

Fig. 3.16. Stable expression of dominant negative form of Rac markedly attenuates mastoparan-induced insulin secretion from INS-1 cells INS-1 cells (control or transfected with dominant inhibitory Rac1) in 24 well-plates were preincubated in KRBH (with 2.8 mM glucose) buffer for 30 min. Then the cells were stimulated by 30 μM mastoparan for 45 min. Results are mean ± SE of three separate experiments in triplicate. ** $P<0.05$ vs. control.

Part II Expression of syncollin affects regulated insulin secretion in INS-1 cells

3.12 Background

Studies on the isolated pancreatic β-cells and established β-cell lines have been extensively carried out to define the underlying mechanism of the regulated exocytosis, which also takes place in exocrine cells, neuronal cells and white blood cells as well. SNARE proteins were originally identified as synaptic proteins but now it is understood that they are universally involved as the core machinery in all intracellular fusion events (Jahn and Sudhof, 1999). It is well known that exocytosis involves these molecules, which are expressed in the above exocytotic cell systems. Consequently, the same mechanism of action of SNARE proteins might be shared in all exocytotic cells. In pancreatic β cells, the v-SNAREs [synaptobrevin (VAMP-2), cellubrevin] and synaptotagmin III are expressed in the secretory granules, whereas the t-SNAREs (syntaxin 1, 2, 3 and SNAP-25) are located on the plasma membrane (Hansen et al., 1999). These SNARE proteins are required for Ca^{2+}-triggered exocytosis of insulin granules since their destruction by proteolysis with specific bacteria toxins abolishes this process (Huang et al., 1998; Lollike et al., 2002; Regazzi et al., 1996b; Wheeler et al., 1996). However, the complete machinery and, in particular, the regulation of this process are still far from clear.

Syncollin was initially identified as a syntaxin-binding protein in pancreatic exocrine acinar cells (Edwardson et al., 1997). Introduction of this protein in an *in vitro* exocytosis system affected the fusion of isolated secretory granules with the plasma membranes, an effect mediated by its interaction with syntaxin in a Ca^{2+}-dependent manner (Edwardson et al., 1997). However, subsequent work found that endogenous

syncollin in exocrine pancreatic acinar cells seems to be a luminal protein in secretory granules and its physiological function remains ambiguous (An et al., 2000). Expression of this protein is also observed in small intestine and parotid gland (Edwardson et al., 1997; Imai et al., 2001; Tan and Hooi, 2000). Interestingly, its expression in small intestine is up-regulated by feeding (Tan and Hooi, 2000), suggesting a potential role in the regulation of secretion of digestion enzymes.

The gene for syncollin isolated from rat expresses a protein of 134 amino acids in full length with a hydrophobic domain at its N-terminal (An et al., 2000; Tan and Hooi, 2000) (Fig.3.17), a common feature of SNARE proteins. On the other hand, the sequence of this hydrophobic domain also meets the criteria as a signal peptide and thus might function to direct the protein into ER lumen to form secretory proteins and ER chaperons. A truncated form of syncollin lack of this domain may result in inappropriately intracellular localization and loss of its normal function.

The findings of potential role of syncollin in exocytotic process *in vitro* and the failure to identify its expression (but cannot rule out the existence of an isoform) in pancreatic β cells, promoted present study to determine the possible role of syncollin in regulated secretion *in vivo* by using intact INS-1 cells as a model, at the time when the first study on this protein was reported (Edwardson et al., 1997).

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5'
1     GTCCATTGTCACC ATG TCC CCG CTG TGC CTG CTG TTG CTG GCT
1                   Met Ser Pro Leu Cys Leu Leu Leu Leu Ala

44    TTA GCC CTG GTG GCT GTC CCC GGT GCC CGA GGC GCT TGT CCA
11    Leu Ala Leu Val Ala Val Pro Gly Ala Arg Gly Ala Cys Pro

86    GTG CCC GCA GAC CTG AAG AAG TCA GAT GGG ACG CGC ACG TGC
25    Val Pro Ala Asp Leu Lys Lys Ser Asp Gly Thr Arg Thr Cys

128   GCC AGG CTC TAT GAG AAC AGT GAC CCC TAC TAT GAC AAC TGC
39    Ala Arg Leu Tyr Glu Asn Ser Asp Pro Tyr Tyr Asp Asn Cys

170   TGC CAG GGG CCT GAA CTG TCT GTG GAT CCA GGC ACC GAC CTG
53    Cys Gln Gly Pro Glu Leu Ser Val Asp Pro Gly Thr Asp Leu

212   CCC TAC CTG CCC TCG GAC TGG TCT AAC TCG GCA TCT TCT CTG
67    Pro Tyr Leu Pro Ser Asp Trp Ser Asn Ser Ala Ser Ser Leu

254   GTA GTG GCC CAG CGC TGC GAG CTC ACC GTG TGG TCT CTC CCT
81    Val Val Ala Gln Arg Cys Glu Leu Thr Val Trp Ser Leu Pro

296   GGT AAA CGT GGC AAG ACA CGC AAG TTC TCT ACG GGT AGC TAC
95    Gly Lys Arg Gly Lys Thr Arg Lys Phe Ser Thr Gly Ser Tyr

338   CCT CGA CTG GAA GAG TAC CGC AAA GGC ATC TTT GGA ACC TGG
109   Pro Arg Leu Glu Glu Tyr Arg Lys Gly Ile Phe Gly Thr Trp

350   GCC AAG TCC ATC TCT GGC CTC TAC TGC AAG TGC TAT TGA TGT
123   Ala Lys Ser Ile Ser Gly Leu Tyr Cys Lys Cys Tyr ***

422   CTTGAGGGTCTGTCGTCTCTGATTCTGCACAGTGCTGAGCCTTACCCAGTCTGTCC

478   TTGGGGATTGCTCTGACCCCAAGAGCCAGGAGGCAAGATGGCCCCATTACCTGCTA

534   TCAGAGCTTCCCTATAATACAGCCCTGGTGTAAGCTAAAAAAAAAAAAAAAAAAAA 3'
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Fig. 3.17 DNA and amono acid sequence of rat syncollin. N-termianl 19 amino acids were deleted in truncated syncollin construct. Myc-tagged constructs was derived by omitting the terminal code TGA when clone the gene into pcDNA/Myc-His vector to get c-Myc tag at C-terminal.

3.13 Syncollin and truncated syncollin display different distribution in subcellular fractions after expressed in INS-1 cells

Syncollin was not expressed in both control INS-1 cell and HIT-T15 cells (Fig. 3.18), as assessed by Western blotting using antiserum against a 16-residue peptide (residues 96-108) of rat syncollin, as well as by RT-PCR by using syncollin N-terminal sequence 5'-accatgtccccgctgtgcct-3' and C-terminal sequence 5'-tcaatagcacttgcagtaga-3' as forward and reverse primers.

After INS-1 cells were stably transfected with the two syncollin constructs through antibiotics (geneticin) selection and verified by western blotting (Fig. 3.18B) and immunofluroscence staining (Fig. 3.19), the polyclonal cell population obtained was used for further studies. In INS-1 cells transfected with syncollin or its truncated form (lacking of N-terminal hydrophobic domain), both cells expressed a protein of approximate 16 kD of molecular mass (Fig. 3.18B, lane 1 and 2).

In this transfected INS-1 β-cell system, both syncollin and its truncated form were found exclusively associated with the membrane-contained subcellular fractions when assessed by Western blotting (Fig. 3.20). In full-length syncollin transfected INS-1 cells, strong signals were detected in the fractions rich of secretory granules or mitochondria and in the plasma membrane, while a weak signal was observed in microsomes (Fig. 3.20). However, in cells transfected with the truncated syncollin, a strong signal was detected in microsomes, a weak signal in fractions rich of mitochondria or secretory granule, but no signal in the plasma membrane. Consequently, syncollin is mainly localized in granule- and mitochondria-enriched fractions as well in the plasma membrane, whereas transfection of the truncated syncollin produces a protein predominantly distributed in microsomes (Fig. 3.20). These results suggest that the N-terminal hydrophobic domain of syncollin may play an important role in directing the expressed syncollin to the intracellular destinations.

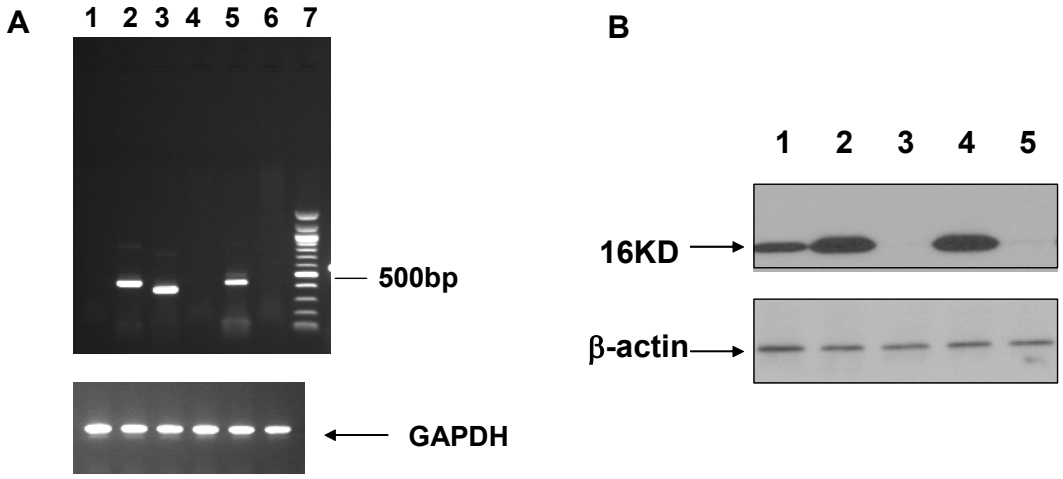


Fig. 3.18 Detection of syncollin expression in insulin secretion cell lines. A. RT-PCR assay of syncollin expression in cell lines and trasnfected cells. Lane1, pcDNA/Myc-His vector tranfected INS-1 cells; lane 2, syncollin transfected INS-1 cells; lane 3, truncated syncollin transfected INS-1 cells; lane 4, INS-1 cells; lane 5, pancreatic acinar cell line AR42J cells as positive control; lane 6, Insulin-secreting HIT-T15 cells. GAPDH was used as internal control. **B.** Western blotting assay of syncollin expression. Lane 1, syncollin transfected INS-1 cells; lane 2, truncated syncollin transfected INS-1 cells; lane 3, INS-1 cells; lane 4, AR42J cells; lane 5, HIT-T15 cells. β-actin was used as internal control.

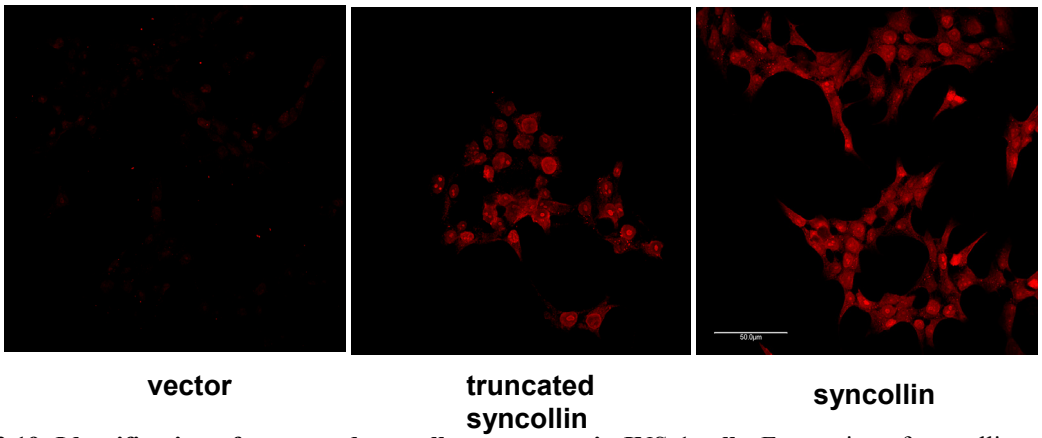


Fig. 3.19. Identification of expressed syncolln constructs in INS-1 cells. Expression of syncollin and truncated syncollin in INS-1 cells was verified by immunofluorescence staining with anti-syncollin antiserum and TRITC-conjugated anti-rabbit IgG. Images were detected by Olympus IX70 Fluoroview 300 laser confocal microscopy. Bar=50μm.

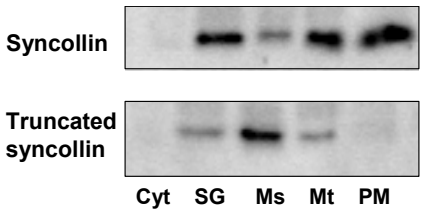
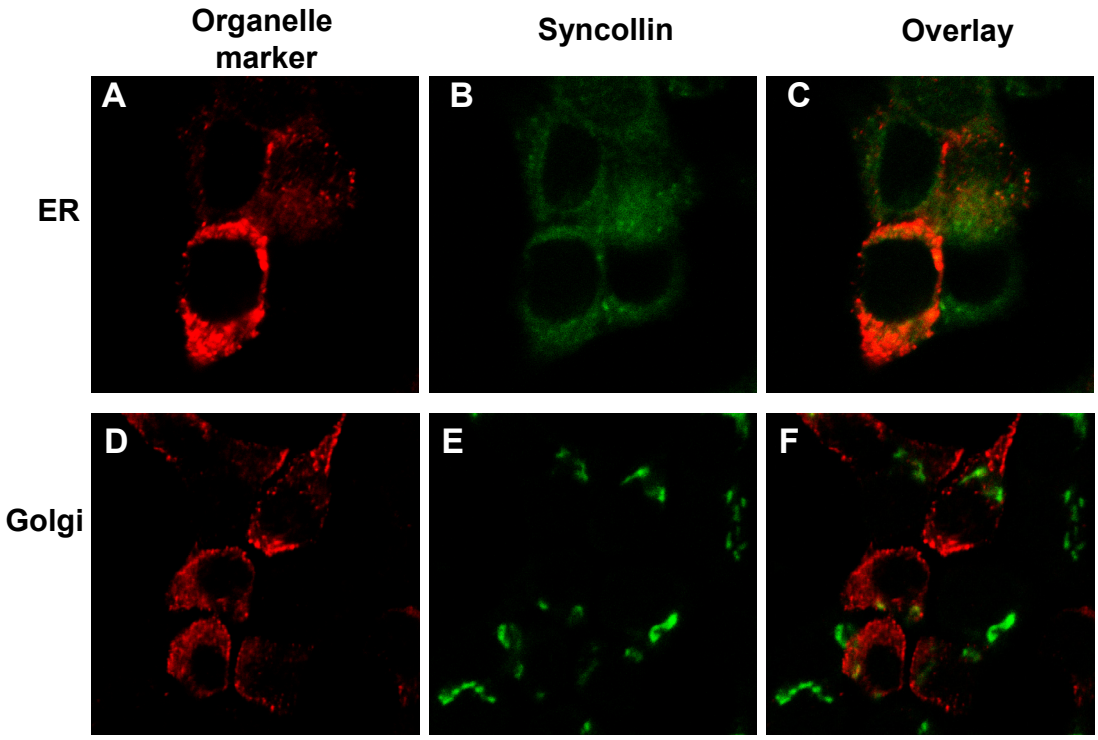


Fig. 3.20. Subcellular localization of expressed syncollin in INS-1 cells by immunoblotting detection. Cells were homogenized and separated by differential centrifugation to obtain cytosol (Cyt), mitochondria-rich (Mt), insulin granule-rich (SG), microsomal (MS), and plasmalemma-rich (PM) fractions. Equal amounts (20 μg) of proteins from each fraction was separated by SDS-PAGE and probed by an anti-syncollin anti-serum. Data are the representative of at least 3 experiments with identical results.

3.14 Syncollin is co-localized with insulin secretory granules, but not ER, Golgi apparatus and mitochondria in INS-1 cells

The subcellular fractionation methods used in this study produce only organelle-enriched, but not sole, subcellular organelle fractions, since analysis of organelle markers in these fractions indicated a cross-contamination between them to various extents (Li et al., 1996). Thus double immunofluorescence staining was also performed in order to better define the intracellular localization of expressed syncollin. The results revealed that, in cells transfected with full-length c-Myc-tagged syncollin, the expressed protein was not co-localized with the markers of ER (calnexin; Fig. 3.21A-C), Golgi apparatus (α-mannosidase II; Fig. 3.21D-F), or mitochondria (MnSOD; Fig. 3.21 G-I). The only co-localized fluorescence signal with syncollin is insulin, which is the marker of secretory granules (Fig. 3.21J-L). As to truncated syncollin, it was found co-localized with the marker of Golgi apparatus (α-mannosidase II; Fig. 3.22D-F), and partly with the marker of ER (calnexin; Fig. 3.22 A-C), but not with the markers of mitochondria (MnSOD; Fig. 3.22 G-I) and secretory granules (Fig. 3.22 J-L). The observed syncollin signal in mitochondria-enriched fraction above by Western blotting could come from contaminated insulin granules, since analyses of the organelle markers in this fraction obtained by differential centrifugation indicated that it contains moderate insulin content (Li et al., 1996). All these observations indicate that transfected syncollin in INS-1 cells is specifically expressed in secretory granules and this process is dependent on the N-terminal hydrophobic domain.



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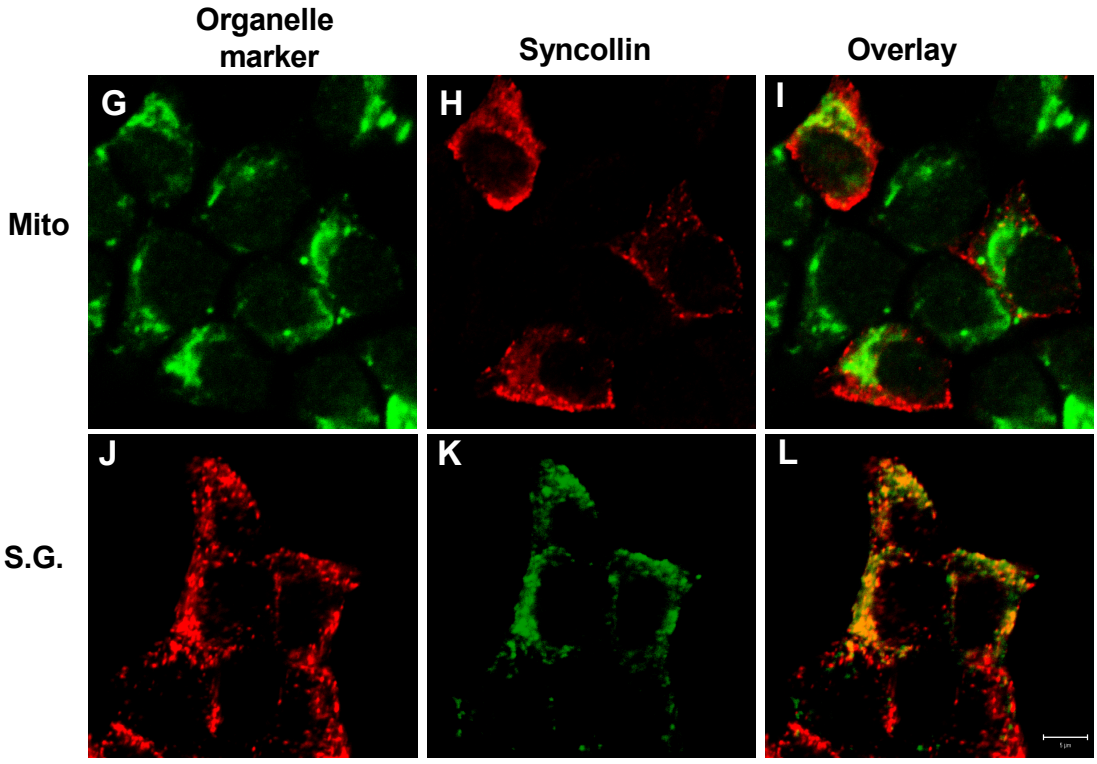
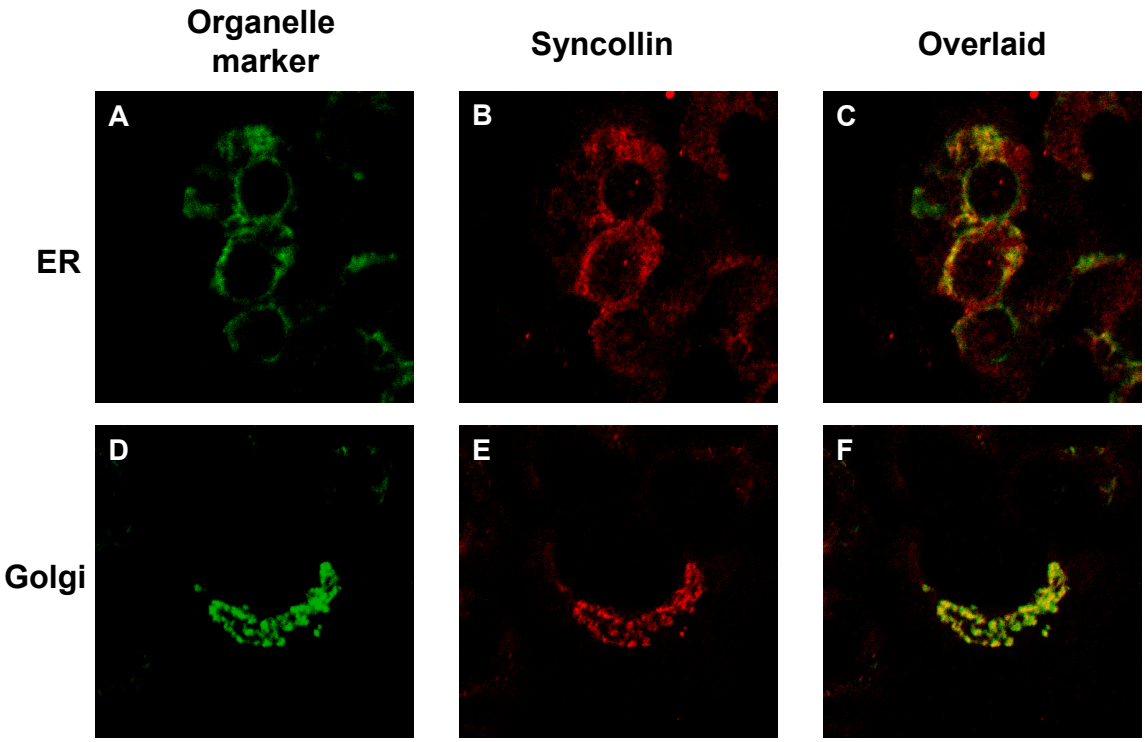


Fig. 3.21. Intracellular co-localization of expressed syncollin in INS-1 cells. Transient expression (2 days) of c-Myc-tagged syncollin in INS-1 cells was examined by immunofluorescence staining with anti-c-Myc monoclonal antibody and TRITC-conjugated anti-mouse IgG (***B, E, H***) or anti-c-Myc polyclonal antibody and FITC-conjugated anti-rabbit IgG (***K***). ER was detected by anti-Calnexin polyclonal antibody and FITC-conjugated anti-rabbit IgG (***A***). Golgi apparatus was detected by anti-α-mannosidase II antiserum and FITC-conjugated anti-rabbit IgG (***D***). Mitochondria were detected by anti-MnSOD polyclonal antibody and FITC-conjugated anti-rabbit IgG (***G***). Secretory granules were detected by anti-insulin monoclonal antibody and TRITC-conjugated anti-mouse IgG (***J***). Images were detected by laser confocal microscopy. Data are the representative of at least 3 experiments with identical results. Golgi, Golgi apparatus; Mito, mitochondria; S.G., secretory granules. Bar = 5 μm.



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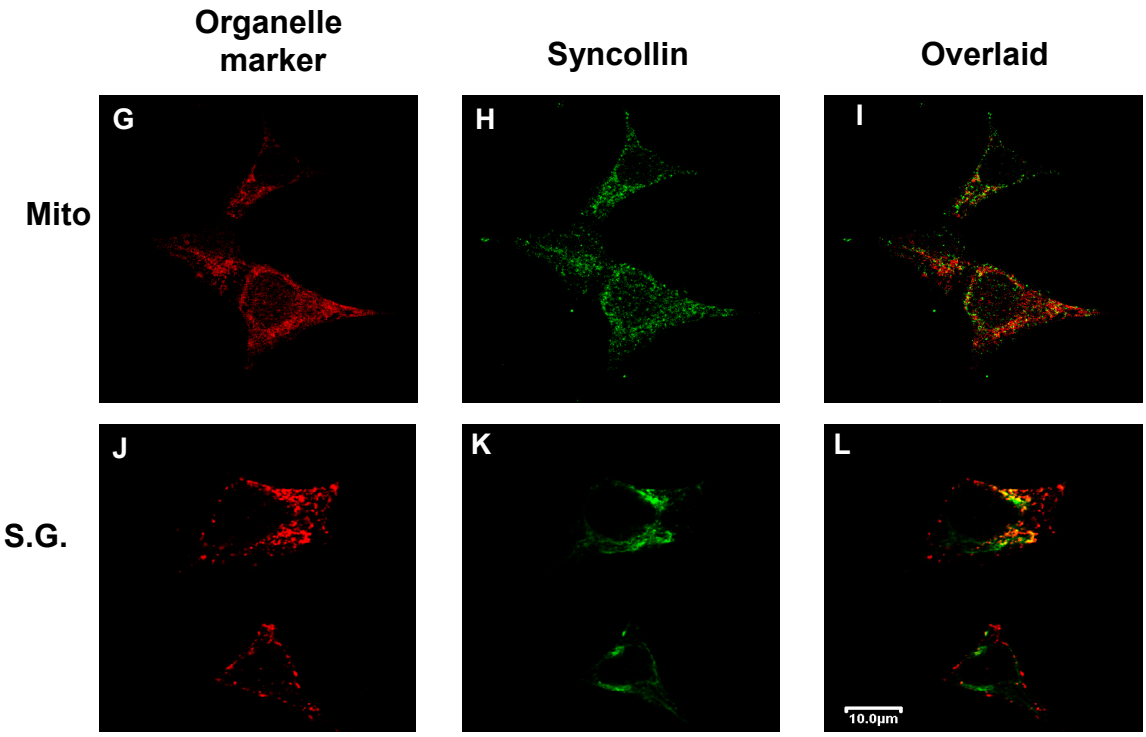

Fig. 3.22. Intracellular co-localization of truncated syncollin in INS-1 cells. Transient expression (2 days) of c-Myc tagged truncated syncollin in INS-1 cells was examined by immunofluorescence staining with anti-c-Myc monoclonal antibody and TRITC-conjugated anti-mouse IgG (***B, E, H***) or anti-c-Myc polyclonal antibody and FITC-conjugated anti-rabbit IgG (***K***). ER was detected by anti-Calnexin polyclonal antibody and FITC-conjugated anti-rabbit IgG (***A***). Golgi apparatus was detected by anti-α-mannosidase II antiserum and FITC-conjugated anti-rabbit IgG (***D***). Mitochondria were detected by anti-MnSOD polyclonal antibody and FITC-conjugated anti-rabbit IgG (***G***). Secretory granules were detected by anti-insulin monoclonal antibody and TRITC-conjugated anti-mouse IgG (***J***). Data are the representative of at least 3 experiments with identical results. Images were detected by laser confocal microscopy. Golgi, Golgi apparatus; Mito, mitochondria; S.G., secretory granules. Bar = 10 μm.

3.15 Insulin release simulated by secretagogues is reduced in INS-1 cells transfected with syncollin but not in cells with its truncated form

Although syncollin in transfected INS-1 cells seems to be localized in secretory granules, the insulin content of INS-1 cells was not altered by stably expressing either syncollin or its truncated form lacking the N-terminal hydrophobic domain (0.41 ± 0.03, 0.42 ± 0.03, and 0.43 ± 0.04 ng /ng DNA, respectively). Moreover, the basal insulin secretion in these cells was also not affected (see the values in Fig. 3.23 legend). However, in full-length syncollin transfected cells, insulin secretion stimulated by 15 mM glucose alone or combined with 1 μM forskolin (which raises cAMP by directly stimulating adenylyl cyclase) was significantly decreased by 47% and 55%, respectively (Fig. 3.23). High potassium (34 mM)-elicited insulin release (through promoting membrane depolarization and Ca^{2+} entry) was also significantly inhibited by 47% in these cells. By contrast, transfection of INS-cells with the truncated syncollin did not alter the insulin secretory responses to the same stimulants above (Fig. 3.23). These results suggest that expression of syncollin in the secretory granules of β-cells impairs insulin secretion upon stimulation, possibly at step(s) as late as Ca^{2+} mobilization or effects.

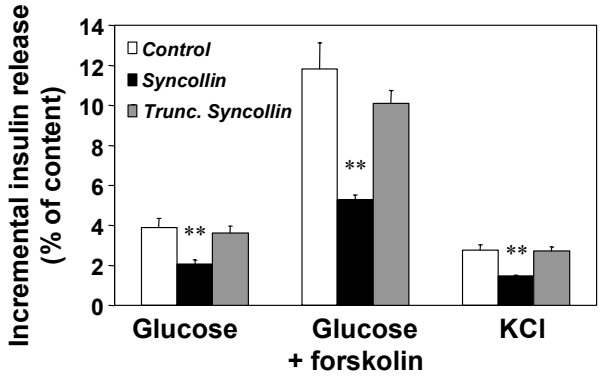

Fig. 3.23. Inhibition of secretagogue-induced insulin secretion in INS-1 cells after transfection of full-length but not truncated syncollin. Cells were cultured in 24-well plates. After stimulation of cells by secretagogues for 30 min, insulin secretion and insulin content were measured by RIA. Basal secretion rates are 2.23 ± 0.19, 2.17 ± 0.21 and 2.82 ± 0.38 (% of content) in control, syncollin-transfected, and truncated syncollin-transfected cells, respectively. Data are mean ± SEM from 4 independent experiments in triplicates. Concentrations used: 15 mM glucose, 1 μM forskolin, 34 mM KC1. ** P<0.01 *vs*. control.

3.16 No effect of syncollin expression on membrane depolarization and $[Ca^{2+}]_i$ elevation

Since the expression of syncollin in INS-1 cells produced a general inhibition on stimulated insulin secretion, the possible alterations in the generation of proximal signals for the stimulus-secretion coupling were examined. The results indicated that glucose-induced membrane depolarization was not altered in INS-1 cells transfected with syncollin (data not shown). Moreover, the $[Ca^{2+}]_i$ elevations evoked by glucose (15 mM) or high K^+ (34 mM) were also not reduced in these cells, although the $[Ca^{2+}]_i$ elevations by high K^+ were slightly (~20%) but significantly higher in the cells transfected by either forms of syncollin (Fig. 3.24). Together these data suggest that syncollin may inhibit insulin at late steps in the exocytotic cascade.

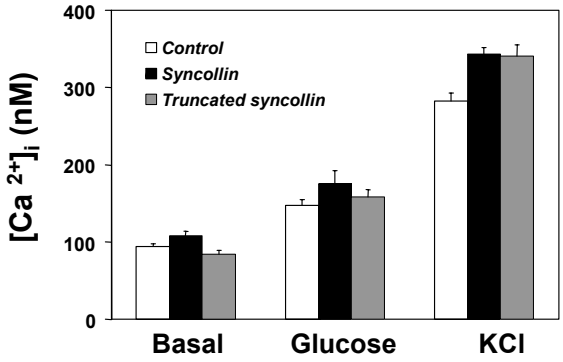

Fig. 3.24. Glucose and high K^+-evoked $[Ca^{2+}]_i$ rises were not reduced in syncollin-expressed INS-1 cells. $[Ca^{2+}]_i$ levels in INS-1 cells were measured by loading a fluorescent Ca^{2+} probe (fura-2) as described in detail in the Methods section. Values are mean ± SEM from 3 independent experiments. Concentrations used: 15 mM glucose, 34 mM KC1.

Chapter 4

Discussion

4.1 The role of Rac1 in regulated insulin secretion

4.1.1 Activation of Rac1 by glucose stimulation in insulin-secreting cells

Earlier studies (Kowluru et al., 1997b; Daniel et al., 2002; Kowluru et al., 2003) have provided indirect evidence for the involvement of Rho subfamily of GTP-binding proteins in physiological insulin secretion. For example, by inhibition of requisite post-translational modifications (i.e., farnesylation, carboxyl methylation, and fatty acylation), it has been shown that blockade of these modifications resulted in a marked reduction of glucose-stimulated insulin secretion from normal rat islets and clonal β cell preparations (Amin et al., 2002; Cheng et al., 2003; Kowluru et al., 1997a). Furthermore, Clostridial toxins (e.g., toxin B and lethal toxin), which selectively glucosylate and inactivate Rho subfamily GTP-binding proteins (Aktories et al., 2000), have been shown to inhibit glucose-stimulated insulin secretion from normal rat islets and clonal beta cells (Kowluru et al., 1997b). In addition, it has been demonstrated that under conditions of stimulated insulin secretion, glucose augmented the carboxyl methylation and membrane-association of Rho family of GTP-binding proteins, including Cdc42 (Kowluru et al., 1997a). Taken together, these findings suggested involvement of Rho GTPases in insulin secretion. Observations in the current study provided the first direct evidence with regard to the identity of one of these GTP-binding proteins as Rac1, since the expression of a dominant inhibitory form of Rac1 resulted in marked reduction in glucose- and cAMP-mediated insulin secretion from INS-1 cells. This action occurred without apparently affecting the secretory response

to elevated $[Ca^{2+}]_i$ *per se*, suggesting that the very distal steps in secretory machinery remain largely intact. The disappearance of F-actin filaments in these cells indicated that F-actin fibers may be necessary for the recruitment of secretory granules to the plasma membrane for exocytosis.

The primary objective of this study was to examine the putative regulatory roles for Rac1, a small GTP-binding protein, in physiologic insulin secretion from insulin-secreting INS-1 cells. The data revealed, for the first time, that physiological concentration of glucose was able to cause Rac1 translocation from the soluble to the membranous fraction, an indication of Rac1 activation. Interestingly, such a glucose-induced translocation of Rac1 could be found only after 15 min stimulation. In addition, direct evidence for Rac1 activation by glucose was demonstrated by measuring the amount of activated, GTP-bound form of the G-protein using a pull-down assay with a GST-PAK-CD as a probe. Although elevating cAMP by forskolin can markedly potentiate glucose-stimulated insulin release, inclusion of forskolin did not enhance glucose-induced Rac1 translocation. Translocation of Rac1 to membranes occurred in other cell systems upon stimulation. A typical example is that in neutrophils stimulated by fMLP or PMA, the cytosolic Rac1 and Rac2 were translocated to cell membranes, a process required for the activation of NADPH oxidase (Dusi et al., 1996). Similar observations were also reported in angiotensin II-stimulated cardiomyocytes and vascular smooth muscle cells (Laufs et al., 2002; Wassmann et al., 2001), in serum-activated MDCK cells (Hansen and Nelson, 2001), and in platelet-derived growth factor stimulated Swiss 3T3 fibroblasts (Fleming et al., 1996).

4.1.2 Altered intracellular distribution of Rac1 mutants and possible relationship with their function

The dominant inhibitory N17Rac1 and constitutively active V12Rac1 are the two Rac mutants most widely used to study the Rac1 function. These two mutants work by different mechanisms on activating and inactivating of Rac1 (Feig, 1999; Ridley, 2000). It is believed that N17Rac1 interferes with the activation of its normal counterpart by competing with the binding to guanine nucleotide exchange factors (GEFs) and thus blocking the functions of endogenous Rac1 (Feig, 1999). In addition, this mutant may not interact with Rac effectors because of a reduction in its affinity to bind to Mg^{2+} which is required for GTPases to reach an active conformation (Feig, 1999). It should be noted that N17Rac1 may also interfere with the functions of other Rho family proteins if these GTPases are activated by the same GEFs.

On the other hand, a mutation of Rac1 at Gly12 (to Val12) decreases its intrinsic and GAP-stimulated GTPase activity, allowing proteins to remain predominantly in the GTP-bound, active form (Ridley, 2000). The mechanisms for the effects of V12Rac1 on cellular functions are less clear and complex. This constitutively active GTPase mutant may directly activate certain Rac1-mediated pathways through interaction with the effectors. However, this active Rac1 mutant presumably may also block the interaction of endogenous Rac1 with downstream targets, since it has been proposed that this mutant can bind to and trap Rac1 effector proteins in the cytosol and prevent their association with target membranes (Feig, 1999). Therefore, the efficiency of this mutant to suppress Rac1 functions would be minimal, whereas its ability to trigger some Rac1-mediated events may be dominant.

Like other Rho proteins, the activity of Rac GTPases depends on their binding to GTP or GDP, which may also govern their intracellular distribution. Thus Rac1 is found in

both cytoplasm and membranes in normal cells (Kraynov et al., 2000; Kumanogoh et al., 2001; Michaelson et al., 2001). In the current study using INS-1 cells, endogenous Rac1 was found mostly localized in cytosol fraction. By contrast, the dominant inhibitory Rac1 mutant was only distributed in the membrane associated fractions enriched in secretory granules and mitochondria. Dominant inhibitory Rac1 could be postulated primarily associated with secretory granules, since the mitochondria-enriched fraction obtained in this study by differential centrifugation may be contaminated by the granules (Li et al., 1996). The constitutively active Rac1 mutant was distributed in cytosol and also all other isolated membrane-containing fractions. The observation that dominant inhibitory Rac1 mutants were predominantly distributed in the membranes seems to belie the notion that, when activated, GTPases would be associated with the target membranes. However, it has been found that mutations that render RhoA, CDC42hs, or Rac1, either constitutively active or dominant inhibitory, abrogated their binding to RhoGDI and redirected them to both plasma and internal membranes in liver cells (Michaelson et al., 2001), which could explain the findings in the present study.

An important observation in this study is that glucose stimulation is able to cause translocation of Rac1 from cytosol to membranes, including plasmalemma, in control INS-1 cells. However, the distribution patterns of either dominant inhibitory or -active Rac1 mutants in transfected cells were not changed when stimulated by secretagogues. Importantly, glucose was no longer able to induce endogenous Rac1 translocation in cells expressing either Rac1 mutants. This failure appears not to be due to the impairment of glucose metabolism and other early steps in stimulus-secretion coupling (e.g. membrane depolarization and elevation of $[Ca^{2+}]_i$), rather it seems more likely due to the interference with endogenous Rac1 by the mutants directly. In addition,

expression of N17Rac1 was also not apparently affecting the secretory response to elevated $[Ca^{2+}]_i$ *per se*, suggesting that the very distal steps in secretory machinery remain largely intact. The inhibitory effects of N17Rac1 could be mediated by two modes: preventing the activation of endogenous Rac1 by competitive binding to GEFs (Feig, 1999) and occupying the target sites of Rac1 on the membranes because of its dominant distribution in the membranes.

Interestingly, expression of V12Rac1 did not enhance basal and stimulated insulin secretion. This is in contrast to the observations in other cells such as mast cells and chromaffin cells, where introduction of constitutively active Rac evoked secretion (Brown et al., 1998; Gasman et al., 1999; Price et al., 1995). Present data suggested that activation of Rac1 may be necessary but not sufficient for the maintenance of stimulated insulin secretion. This notion is also supported by the observation that expression of wild-type Rac1 did not affect both cell morphology (data not shown) and secretory responses.

The non-specific effects due to overexpression of a protein in transfection experiments should be considered. There is evidence that such an effect was minimal in this study. No overexpression was found in stable transfected cells, since the total amount of Rac1 (endogenous plus mutant) from cell lysate of either Rac1 mutant-transfected cells was not significant different from that of control cells. Only small amount of mutant proteins was found in contrast to endogenous Rac1. And marked alterations of insulin secretory response, cell morphology and F-actin cytoskeleton were only observed in cells transfected with N17Rac1, but not V12Rac1, suggesting a specific effect of the former. Furthermore, stable expression of wild type Rac1 had no effect on morphology and secretagogues-induced insulin release in INS-1 cells.

4.1.3 Involvement of Rac1 mainly in the late phase of insulin secretion

It is well known that glucose induces biphasic insulin secretion (Bratanova-Tochkova et al., 2002; Henquin et al., 2002; Rorsman et al., 2000). Accumulating evidence favorsthe two-compartmental model (Bratanova-Tochkova et al., 2002; Rorsman et al., 2000), in which the second phase of secretion requires the promotion/recruitment of secretory granules to the exocytotic sites, although the details of underlying mechanisms remain to be defined. Current studies revealed that only the late phase of glucose-stimulated insulin secretion was significantly reduced in N17Rac1-transfected INS-1 cells. Moreover, although both phases of insulin secretion induced by forskolin plus glucose were attenuated in these cells, a larger inhibitory effect occurred during the late phase. These results suggest that Rac1 may play a major role in the late phase of glucose-stimulated insulin secretion. That fact that glucose only significantly increased Rac1 translocation to membranes at 15 min also supports this notion. The inhibitory effect on insulin secretion by N17Rac1 might be attributable to the interference with glucose-induced activation of endogenous Rac1 as discussed above.

Although an involvement of Rac proteins in the Ca^{2+}-dependent exocytosis has been reported in neuronal cells (Doussau et al., 2000; Komuro et al., 1996), expression of the inactive Rac1 mutant did not significantly affect the insulin secretion stimulated by Ca^{2+} *per se* (through high K^+ depolarization) in INS-1 cells. Thus it seems that Rac1 did not engage in the very distal step of exocytosis in INS-1 cells. In addition, the preferred inhibition of the late phase secretion by the expression of N17Rac1 may also explain its failure to significantly inhibit insulin secretion induced by high K^+, since only a monophasic insulin release (equivalent to the early phase) was evoked under this stimulatory condition (Sato et al., 1992).

4.1.4 Actin cytoskeleton reorganization may contribute to Rac1 effects on the maintenance of morphology and regulation of insulin secretion in β-cells

There is strong evidence that Rac is a key control element in the reorganization of actin cytoskeleton (Hall, 1998). Microinjection of V12Rac was capable of inducing lamellipodia and membrane ruffle as well as subsequent stress fiber formation, whereas injection of N17Rac prior to the addition of growth factors or together with V12Rac abolished these effects in fibroblasts (Ridley et al., 1992). Thus it is not surprising that the morphology of β-cells would be altered by interference with Rac function. The morphological changes in Rac1 mutants-transfected cells were accompanied with the alterations in the F-actin distribution. F-actin filaments almost completely disappeared in N17Rac1-transfected cells as assessed by phalloidin staining and these cells were rounding-up. Surprisingly, expression of constitutively active Rac1 mutant did not enhance the formation of actin cytoskeleton as in other cell types (Ridley et al., 1992), rather had small opposite effect. Importantly, F-actin structure may be not only for the maintenance of INS-1 cell morphology but also involved in the transport of secretory granules, since loss of these F-actin filaments in N17Rac1-transfected cells displayed preferred inhibition of the late phase of stimulated insulin secretion. Previous study using *Clostridium botulinum* C2 toxin to disrupt F-actin structure also found a more severe inhibition of the second phase of insulin secretion in HIT-T15 cells and in isolated pancreatic islets (Li et al., 1994). Together, these findings suggested that the role of Rac1 in regulated insulin secretion may involve the regulation of actin cytoskeleton reorganization for secretory granule recruitment which is required for the second phase of secretion (Howell and Tyhurst, 1986; Li et al., 1994).

4.1.5 Rac may be involved in cAMP potentiated insulin secretion

It is well known that protein kinase A (PKA) activation by cAMP can positively influence regulated secretion in many cell systems including β-cells. It has been reported that PKA can regulate Rac1-dependent organization of actin cytoskeleton during the migration of carcinoma cells (O'Connor and Mercurio, 2001). It is thus likely that Rac1 inactivation may affect the PKA-mediated potentiation of insulin secretion through disruption of actin cytoskeleton organization in INS-1 cells. Indeed the late phase of forskolin-potentiated insulin secretion was more heavily inhibited in N17Rac1 transfected cells, suggesting that forskolin-activated PKA may be important for the trafficking of secretory granules in β-cells (Hisatomi et al., 1996; Renstrom et al., 1997). On the other hand, forskolin may also potentiate insulin secretion by PKA-independent mechanism through cAMP-GEFII which interacts with Rap1 (belong to Ras family) and Rim proteins (Rab3-interacting molecules) (Ozaki et al., 2000; Renstrom et al., 1997), indicating the existence of a cross-talking between cAMP and small G-proteins. Whether cAMP is able to activate GEFs for Rho family proteins or/and interact with them remains to be defined, though a clear effect of forskolin on Rac1 translocation in INS-1 cells could not be detected in the present study.

4.1.6 Role of Rac1 in mastoparan-induced insulin secretion from β-cells

Mastoparan is capable to stimulate insulin secretion by activating G-proteins, presumably via activation of trimeric G proteins. However, there is also evidence from collaborated work for the implication of Rac in this scenario by using Clostridium toxins to inactivate certain members of Rho proteins (Amin et al., 2003). Mastoparan-induced insulin secretion was attenuated in INS-1 cells expressing the dominant inhibitory Rac1 mutant, though less extent than the glucose and forskolin-stimulated

secretion, suggesting the involvement of other molecules. Mastoparan is able to activate Rac1 in β-cells, as demonstrated by direct measurement of active Rac1 and translocation of Rac1 (also Cdc42 to a less extent) from cytosol to insulin-containing granules (Amin et al., 2003). Interestingly, mastoparan-induced GTP/GDP exchange of Rac did not require post-translational modification of C-terminal cysteins (Ohara-Imaizumi et al., 2001), which was essential for glucose-induced insulin secretion (Amin et al., 2003). In addition, expression of wild type Cdc42 increased mastoparan-induced insulin secretion in an insulin-secreting cell line (βHC9) (Daniel et al., 2002), suggesting other Rho family proteins may be also involved in mastoparan stimulated insulin release. Therefore, it is likely that Rac/Cdc2 translocation to insulin granule is essential for mastoparan-induced insulin release (Amin et al., 2003; Daniel et al., 2002). Whether Cdc42 is involved in glucose-stimulated insulin secretion remains to be investigated.

4.1.7 PIP5K may play a role downstream of Rac1 in regulated insulin secretion

Phosphatidylinositol-4 phosphate 5 kinase (PIP5K) is a key enzyme catalyzing the synthesis of phosphatidylinositol-4,5-bisphosphates [PtdIns(4,5)P_2] and a potential effector of Rac1. PtdIns(4,5)P_2 is important for signal transduction, cytoskeletal and membrane trafficking events (Martin, 1998). A decrease in PtdIns(4,5)P_2 levels caused inhibition of exocytosis in neuroendocrine cells, suggesting that PtdIns(4,5)P_2 synthesis can influence exocytosis either by interacting with SNARE proteins directly or by anchoring ADP-ribosylation factor and GAPs to the fusion site of membrane (Martin, 1998). There are several PIP5K isoforms, including the 68-kDa type I (PIP5KIα and PIP5KIβ) and 53-kDa type II (PIP5KII). PIP5KIs have wide tissue distributions, but their expression levels differed greatly. PIP5KIs are stimulated by phosphatidic acid and possibly regulated by Rac G-proteins since they could directly

associate with Rac1 but not Rho and Cdc42 (Tolias et al., 2000). GTPγS has been found to increase PIP5K activity in many tissues and cells (Stephens et al., 1993). Rac activation causes an increased synthesis of PtdIns(4,5)P_2, which may be mediated by Rac stimulation of a PIP5KIs (Chatah and Abrams, 2001). The downstream proteins in this scenario are gelsolin and other capping proteins whose actin barbed-end capping activities are inhibited by phosphoinositides. In platelets, Rac activation results in increased actin polymerization for lamellae formation, which arises from an increase in the number of free actin barbed-ends available for filament elongation due to PtdIns(4,5)P_2 binding to cap proteins (Tolias et al., 2000). Thus PIP5KIs may function as an effector of Rac in mediating the reorganization of actin cytoskeleton.

It is interesting that PIP5K can associate with both GTP- and GDP-Rac either *in vitro* or *in vivo* (Martin, 1998; Tolias et al., 2000). PIP5K binds to the C-terminus of Rac and this interaction is augmented in the presence of specific phospholipids (Carpenter et al., 1999). Since PIP5K activity is not affected by Rac *per se* (Chong et al., 1994), the effect of Rac1 on exocytosis by regulating PIP5K is most probably achieved by promoting its translocation. It seems that PIP5KI, not PIP5KII, mediates Rac-dependent actin assembly and is required for secretion (Tolias et al., 2000). The results from current study revealed that incubation with 15 mM glucose significantly increased translocation of PIP5KIα (~60 kD) from cytosol to the membranes in INS-1 cells and such an effect was not demonstrable when the cells were transfected by a dominant inhibitory Rac1 mutant. These results are very similar to those with Rac1, suggesting the increased PIP5KIα translocation to membranes is possible due to its binding with Rac1. Thus a close relationship between the two proteins and their association with insulin secretion may exist in β-cells. The interference with PIP5KIα translocation to the membrane might contribute to the observed disruption of actin

cytoskeleton and inhibition of insulin secretion in the N17Rac1-transfected INS-1 cells, since this may decrease the local concentrations of PtdIns(4,5)P_2, which is required for ATP-dependent priming in neuroendocrine cells (Martin, 1998).

In conclusion, glucose stimulation increased Rac1 activity and led to its translocation from cytosol to the membranes in INS-1 cells. Expression of dominant inhibitory Rac1 abolished such effect. This study also provided direct evidence for a role of Rac1 in the regulation of insulin secretion, as demonstrated by the clear inhibition of glucose- and forskolin-induced insulin secretion in INS-1 cells expressing negative Rac1. Such inhibitory effect was mainly on the late phase of glucose and forskolin stimulated insulin secretion. The disappearance of F-actin filaments in these cells indicated that these F-actin fibers may be necessary for the recruitment of secretory granules to the plasma membrane for exocytosis. This action was not associated with an impairment of generating proximal signals required for glucose-evoked secretion. It seems that Rac1 is not involved in the very distal steps in secretory event, since the secretory response to elevated $[Ca^{2+}]_i$ *per se* was affected in N17Rac-transfected cells. In addition, mastoparan (which activates Rac1)-induced insulin secretion was also inhibited in INS-1 cells expressing N17Rac1. It is possible that PIP5KIα, which is implicated in actin cytoskeletal organization and secretory event, may be an effector of Rac1 participating in glucose-induced insulin secretion.

4.2 Intracellular targeting of syncollin and its possible role in regulated secretion

4.2.1 Expressed syncollin is associated with membranes in INS-1 cells

In current study, syncollin and its truncated form (lack of signal peptide) were stably expressed in a widely used insulin-secreting β-cell (INS-1) line. The expressed products from transfection of both syncollin and its truncated form were membrane-associated, when assessed by subcellular fractionation followed by Western blotting. These observations seem puzzling at glance, since the truncated syncollin does not contain a membrane-associated domain. Moreover, the N-terminal hydrophobic domain in the full-length syncollin transfected INS-1 cells is apparently severed during the processing after translation, since the molecular mass of detected proteins in these cells was identical to that in the cells transfected with the truncated form lack of the domain (Fig. 3.16A). In the course of carrying out this study, the biochemical properties of syncollin were further characterized by other laboratories. A series of studies on pancreatic acinar cells revealed that the full-length syncollin has a cleavable signal sequence and that syncollin can be washed away from granule membranes by sodium carbonate (An et al., 2000). The native syncollin appeared to be associated with membrane lipid microdomain in zymogen granules and such membrane association involves its interaction with cholesterol (Geisse et al., 2002; Hodel et al., 2001). In addition, syncollin may form a pore structure as homo-oligomers (Geisse et al., 2002; Hodel et al., 2001), oligomerization of which may produce a hydrophobic region that can render syncollin membrane-associated.

4.2.2 N-terminus of syncollin is essential for its sorting to secretory granules

The presence of the signal peptide-like sequence (N-terminal hydrophobic domain) in syncollin during translation indicates that syncollin could be directed to endoplasmic reticulum for further process such as forming homo-oligomers. Endogenous syncollin in exocrine acinar cells was found to be a secretory granule associated protein (Edwardson et al., 1997). In transfected INS-1 β-cell system, the products of expression of both syncollin and its truncated form (lacking of N-terminal hydrophobic domain) are associated with the membranes. However, immunofluorescence studies revealed different intracellular targeting: a co-localization of syncollin staining specifically with insulin (indicative of secretory granules) in cells transfected with full-length syncollin, but not in cells transfected with a truncated form. In the case of the latter, expressed syncollin is co-localized mainly with Golgi apparatus and also partly with ER. These results suggest that the expressed syncollin in INS-1 cells can be sorted to secretory granules as occurred in pancreatic and lacrimal acinar cells (Jerdeva, 2003; Tan and Hooi, 2000). However, although truncated syncollin is also associated with membranes possibly due to forming a structure with hydrophobic property though oligomerization, the targeted location is different from insulin granules. It is apparent that the N-terminal hydrophobic domain of syncollin serves as the signal peptide in this case and is essential for syncollin sorting to secretory granule in both exocrine and endocrine cells. In a study examining the intracellular targeting of syncollin by Hodel and Edwardson, it was found that both GFP- and His(6)-tagged (at C-terminal) syncollins were sorted to the vesicles in both AtT-20 cells and AR42J cells (Hodel et al., 2001). Interestingly, His(6)-tagged syncollin was efficiently targeted to secretory granules only in the former but not in latter cells, whereas GFP-tagged syncollin was identified in the secretory granules of both types of cells (Hodel et al.,

2001). In lacrimal acinar cells, syncollin was found mainly in Rab3D-enriched secretory vesicles and less in VAMP2- enriched secretory vesicles (Jerdeva, 2003). In addition, carbachol stimulation altered the syncollin distribution and syncollin-associated vesicles were concentrated underneath the plasma membrane (Jerdeva, 2003).

4.2.3 Expression of syncollin does not affect insulin content and secretagogue-evoked $[Ca^{2+}]_i$ increases

Expression of syncollin in INS-1 cells did not alter insulin content, suggesting insulin synthesis/processing is not impaired in these cells. Importantly, glucose-induced membrane depolarization and $[Ca^{2+}]_i$ rises were not affected. $[Ca^{2+}]_i$ elevation evoked by high K^+ (which artificially causes membrane depolarization without generating metabolic signaling coupling factors) also remained not reduced. These data indicate that the proximal stimulus-secretion coupling signals are not affected by transfection of syncollin in INS-1 cells.

4.2.4 Syncollin on the granules inhibits secretagogue-induced insulin secretion

Syncollin was originally found as a Ca^{2+} regulated syntaxin-binding protein capable of affecting exocytosis in a cell-free system in an inhibitory manner (Edwardson et al., 1997). In current study, its possible role in regulated secretion in an exocytosis cell system was investigated. The resultant data revealed that syncollin expression in INS-1 β-cells inhibited stimulated insulin secretion and such inhibitory effect occurred in the distal stage of secretion process, since insulin secretion evoked by all stimulants including high K^+ (presumably a pure Ca^{2+} stimulation) was impaired. The fact that inhibition of stimulated insulin secretion only occurred in the cells transfected with full-length syncollin, but not in the cells with the truncated syncollin missing the signal

peptide, indicated that syncollin sorting into secretory granules is essential for affecting regulated insulin secretion. Transfection of syncollin in rat islets also inhibited insulin secretion as reported by other laboratories (Hays, 2002).

The inhibitory effect of syncollin on stimulated insulin secretion may be related to its specific location, its molecular property, and its ability to interact with syntaxin. First, inhibition of insulin secretion only occurs in cells in which syncollin is localized in the secretory granules, a target partner involved in secretion event. Second, the strong membrane association and possible ability to permeabilize the bilayer membrane in the form of homo-oligomers make it possible to interact with other granule-associated proteins in secretory granules. For instance, syncollin-associated membrane rafts contain not only GP-2, a major glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein, which is believed to be a sortase, but also the members of v-SNAREs such as synaptobrevin-2 (Hansen et al., 1999). Third, syncollin may interact with non-granule-associated, or plasmalemma-associated t-SNAREs such as syntaxin (Hansen et al., 1999; Tan and Hooi, 2000), since syncollin may be exposed to the granule outer surface due to its permeabilizing feature (Geisse et al., 2002). This could impede the normal interaction between t- and v-SNAREs as well as SNAREs and other regulatory molecules. However, these postulations remain to be verified by experimentally. A very recent study expressing of syncollin in AtT-20 neuroendocrine cells also displayed similar inhibitory effect on regulated secretion of corticotrophin possibly by inducing uncontrolled permeabilization and destabilization of secretory granules (Walse et al. 2004). However, it is still unclear whether the interference with stimulated secretion by syncollin is resultant of impairment of a step at insulin granule recruitment, docking, priming, or fusing.

4.2.5 Is there any physiological role of syncollin in insulin secretion in β-cells?

The physiological function of syncollin is not well defined. Beside existence in pancreatic acinar cells, syncollin was also found in small intestine, parotid gland and lacrimal acinar cells (Edwardson et al., 1997; Jerdeva, 2003; Tan and Hooi, 2000), and its expression was inducible (Tan and Hooi, 2000). All these observation implicate that syncollin is a widely expressed protein related to the secretion of digestion enzymes in exocrine cells. The sorting of syncollin into zymogens suggests that syncollin may be involved in the processing of zymogen contents in exocrine cells. However, the syncollin-knockout mice did not display any abnormal phenotype either in zymogen contents or in regulated secretion, and only exhibited reduced stress responses (Antonin et al., 2002). Nonetheless, its possible physiological role in regulated secretion cannot be completely excluded, since a compensatory effect may occur, which is observed in many knockout cases. Moreover, syncollin homologue proteins may exist in islet β-cells. Indeed, Rhodes *et al* have identified a syncollin-like protein called phosphohipolin in β-cells (Rhodes, 2002). Therefore, it is possible that the observed alterations of secretory responses from expressing syncollin in the INS-1 cell experimental system are due to either an action of itself or the interference with the endogenous homologue. Nevertheless, INS-1 cells expressing syncollin may be used as a model cell system to examine its function in the cell.

In conclusion, the results from this study revealed that insulin secretion induced by several well-known secretagogues was inhibited by expression of syncollin in INS-1 β-cells. The data also indicate that sorting of syncollin to secretory granules is necessary to affect the stimulated insulin secretion, an event dictated by the N-terminal hydrophobic domain of the protein. There was no alteration of insulin content nor impairment in the generation of the proximal signals (membrane depolarization and

increase of $[Ca^{2+}]_i$) required for triggering insulin secretion, pointing to an action on the distal steps in exocytosis. However, physiological function of syncollin, in particular its possible interaction with SNAREs in the intact cell, needs to be further clarified.

4.3 Future work

The results from above work provided evidence for the involvement of Rac1 and syncollin in the regulated exocytosis in insulin-secreting INS-1 cells. It is found that glucose stimulation could activate Rac1 and this event was essential, but not sufficient, for glucose and forskolin stimulated insulin release. The data also suggested that Rac1 activation might influence the regulated insulin release via an effect on actin reorganization which is required for the granule recruitment. However, the effector(s) which links the activated Rac1 to the process of actin reorganization and exocytosis is still unclear in β-cells. The preliminary results revealed that PIP5K-Iα, an effector of Rac1 (Tolias et al., 2000), was translocated from cytosol to the membranes following glucose stimulation in INS-1 cells, an event correlated with Rac1 translocation. Thus, further work needs to be done on PIP5K-Iα by knocking down this protein (e.g. siRNA) to see any change in secretory responses. The other possible effector of Rac1 worthy of examination is p21-activated kinase (PAK), which mediates actin reorganization (Sells et al., 1997) and may participate in other processes. I have identified PAK-α, but not PAK-γ, isoform in INS-1 cells. Knock down of PAK may improve our understanding how Rac1 activation regulates glucose and forskolin stimulated insulin secretion. In addition, the current study by transfection of dominant inhibitory Rac1 cannot completely rule out the role of other Rho proteins such as

Cdc42 in glucose-induced insulin secretion, since both Rac1 and Cdc42 share and may bind to some common GEFs competitively (Feig, 1999). Specifically inactivation or knock down of cdc42 may help answer this question.

Although syncollin might not play a physiological role in regulated insulin secretion in β-cells, the latter cell system provides a model to investigate the intracellular targeting and interaction of syncollin with other proteins in the cell. This study has found that the N-terminal hydrophobic domain is necessary for syncollin sorting into secretory granules and expression of syncollin affects the regulated insulin secretion at a distal step of exocytosis in INS-1 cells. However, the mechanism underlying inhibition of insulin secretion by syncollin is not clear. The possibility of translocation (flip) of this protein from the inner of granule membrane to the out surface of granules, where it may interact with syntaxin, is required to be explored. Yeast two hybrid system can also be useful to screen the interacting proteins of syncollin, especially those involved in distal step of exocytosis. In addition, acute knockdown of syncollin by siRNA transfection in the cells such as pancreatic acinar cells that express syncollin will help understand the possible physiological role of syncollin in exocytosis.

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Appendix
(additional data)

The additional data below are not included in the main text of this thesis.

A1. Expression of dominant inhibitory Rac1 affected cell size and cell growth of INS-1 cells

Dominant inhibitory Rac1 transfected cell displayed multiple varieties beside its inhibitory effects on stimulated insulin secretion. Detected by flow cytometry, the diameter of these cells was smaller than that of control cells (9.9 ± 0.5 vs. 12.6 ± 0.7 nm) (Fig. 1). No change of cell size was found in constitutively active V12Rac1 transfected cells. The proliferation rate of N17Rac1 transfected cells was retarded by ~30% after 6-day culture, and this effect became significant at 2 day culture (Fig. 2). The proliferation rate of constitutively active V12Rac1 transfected cells increased modestly in comparison with control cells at 6 day culture. Cell cycle analysis by flow cytometry displayed an increase in the number of cells arrested in G2/M phases in N17Rac1 transfected cells (21.3% vs. 5.8% of total cells); however no apoptosis was found in these cells (Fig. 3). Constitutively active Rac1 had no such effects on INS-cell growth. The decrease of cell size in N17Rac1 transfected cells may result from the inhibition of actin polymerization, which is essential for the maintenance of cell morphology. G2/M arrest caused by expression of dominant inhibitory N17Rac was also found in fibroblasts (Moore et. al., 1997). This effect on cell cycle is probably due to interference of Rac1 interaction with its effectors in mitogenic pathway, and may be

the reason for cell growth retardation in these cells. These data indicated that expression of dominant inhibitory Rac1 may inhibit cell growth.

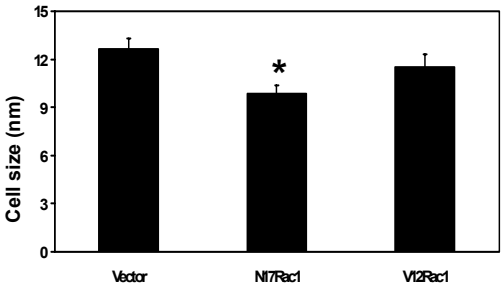

Fig. 1. Cells size of INS-1 cells expressing Rac1 mutants. INS-1 cells were cultured in 6-well culture plate before trypsinized and resuspended in 1 ml culture medium. Cell size was determined by flow cytometry. The cell size was indicated as diameter. Data are the mean ± SEM of 3 observations. * P<0.05 *vs*. control.

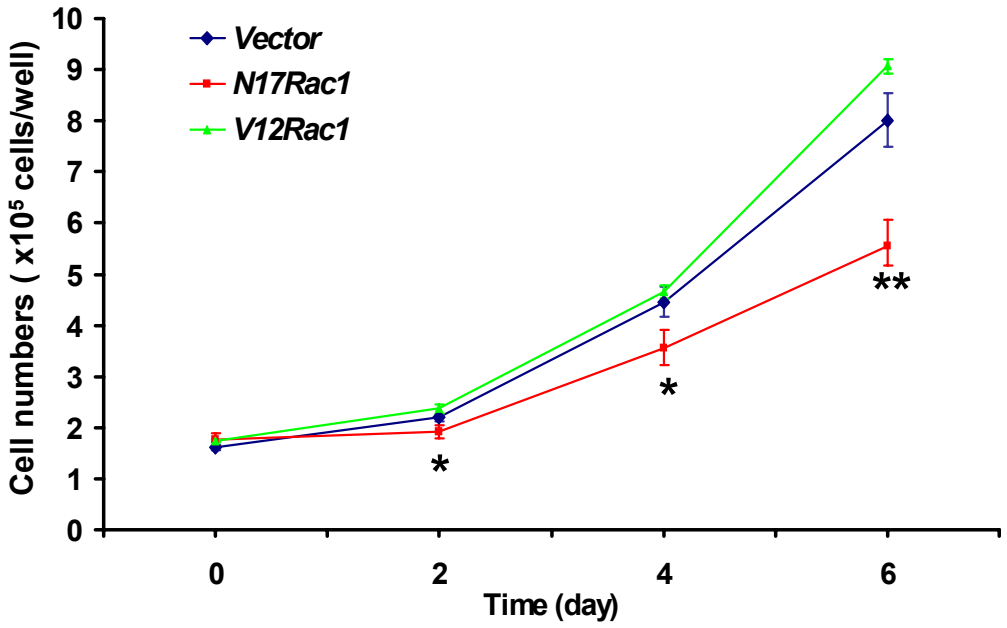

Fig. 2. Proliferation of INS-1 cells expressing Rac1 mutants. The number of INS-1 cells was counted at 0, 2, 4, 6 day after subculture. Data are the mean ± SEM of 3 observations. * P<0.05, ** P<0.01 *vs*. control.

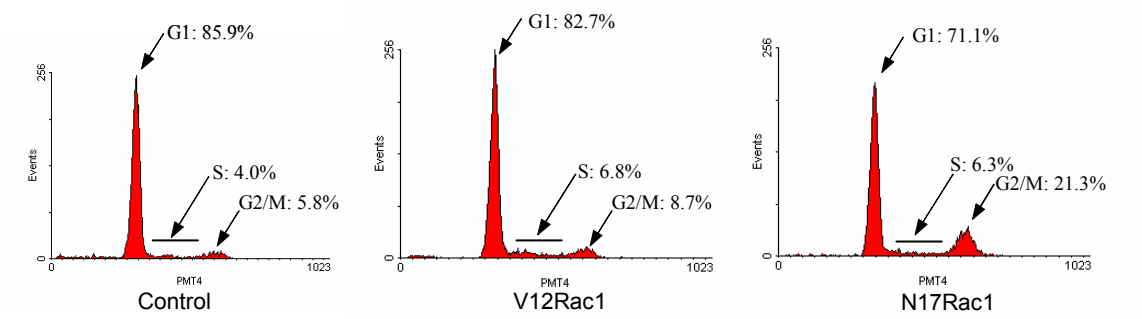

Fig. 3. Flow cytometric analysis of INS-1 cells expressing Rac1 mutant. INS-1 cell cultured for 3 days were examined by propidium iodide (PI) staining and flow cytometric analysis. The cells at different cell cycle stages were presented as the percentage of total analyzed cells. (Acknowledge to Dr. Huo Jianxin for helping to perform the experiment)

A2. Both dominant inhibitory and constitutively active Rac1 expression reduced F-actin content in INS-1 cells

Although the F-actin staining by rhodamine-phalloidin displayed significant decrease in dominant inhibitory Rac1 transfected INS-1 cells, there was only modest signal decrease in constitutively active Rac1 transfected cells. To quantify the extent of F-actin decrement in Rac1 mutant transfected cell, the F-actin content was detected by rhodamine-phalloidin staining followed with methanol extraction of the F-actin bound dye and rhodamine fluorescence signal was detected by SpectraMax Gemini fluorescence plate reader (Molecular Device). The F-actin content was decrease in both Rac1 mutant transfected cells. There was 44% decrement of signal in cells expressing N17 Rac1 and 39% in cells expressing V12 Rac1 (Fig. 4). The decreased signal for F-actin content in cells expressing V12Rac1, although not significant by immunofluorescence staining, might be due to the interference caused by V12Rac-induced activation of actin binding protein confilin and gelsolin that were known

regulated by Rac (Stanyon and Bernard, 1999; Azuma et. al., 1998), rather than inhibition of action polymerization. Confilin and gelsolin could bind to F-actin and block phalloidin binding either by change F-actin structure (McGough et. al.1997) or by competitively binding to phalloidin bound position (Allen and Janmey, 1994). The results indicated that dominant inhibitory and constitutively active Rac1 interfere with F-action polymerization by different mechanisms.

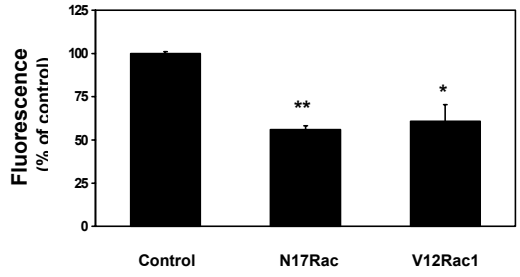

Fig. 4. F-actin content in Rac1 mutant transfected INS-1 cells. F-actin content was assayed by rhodamine-phalloidin staining and fluorescence intensity was examined by SpectraMax Gemini plate reader. Data are the mean ± SEM of 4 observations. * $P<0.05$, ** $P<0.01$ *vs*. control.

A3. Glucose stimulation did not induce Rac1 translocation to F-actin filaments

To investigate the target of Rac1 translocation induced by glucose stimulation in INS-1 cells, immunofluorescence staining and confocal microscopy were used to study the co-localization of Rac1 and F-actin in these cells. When stimulated by 15 mM glucose, no Rac1 and F-actin co-localization was found in the cells in either control cells (Fig. 5A) or transfected cells (Fig. 5, B and C). The results suggested that glucose stimulation could not induce Rac1 translocated to F-actin filaments. The failure of Rac1 to co-localize with F-actin filaments spatially may be because Rac1 regulates actin polymerization through its downstream effector (Van Aelst and D'Souza-Schorey, 1996).

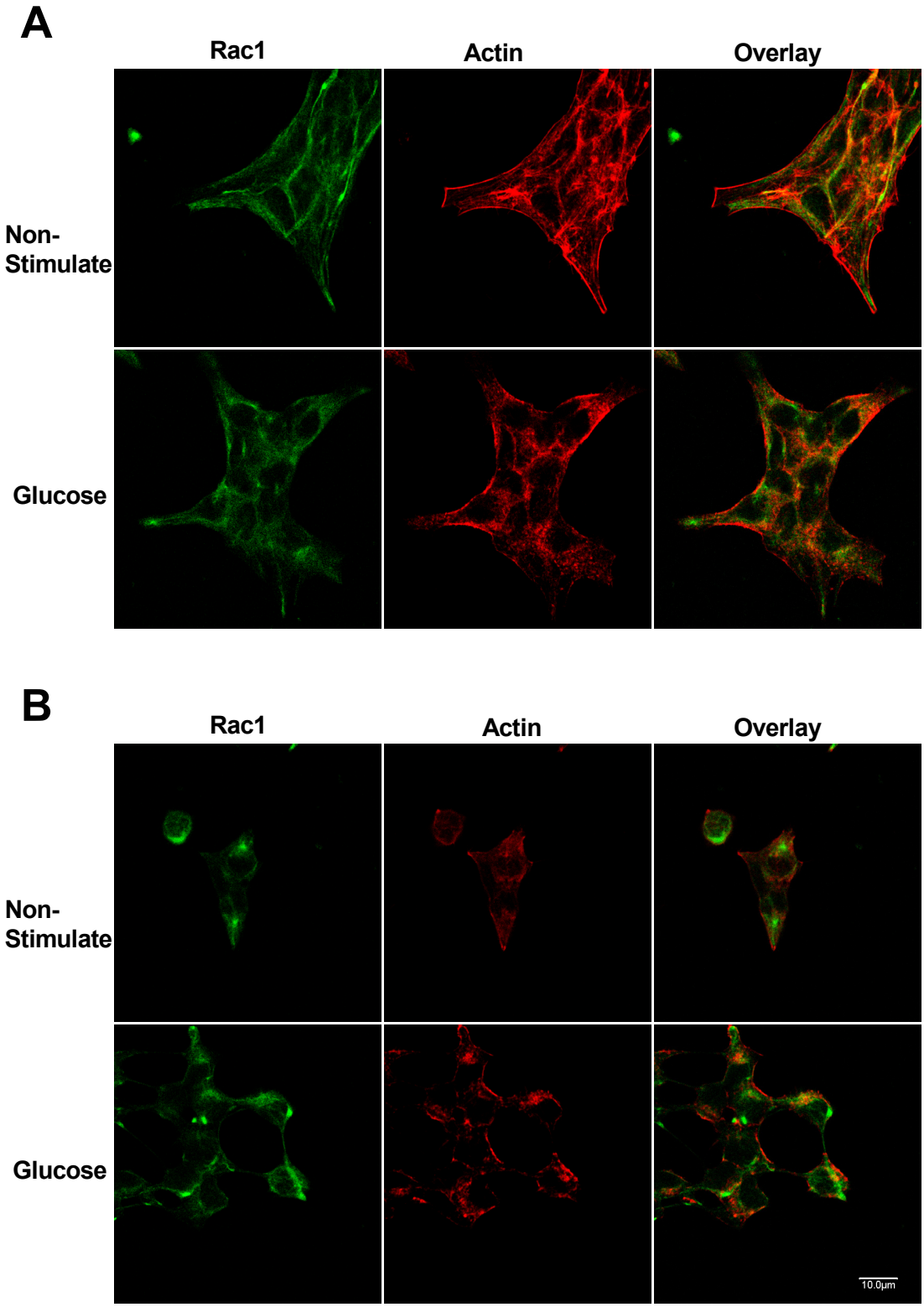

Fig. 5. (continue to next page)

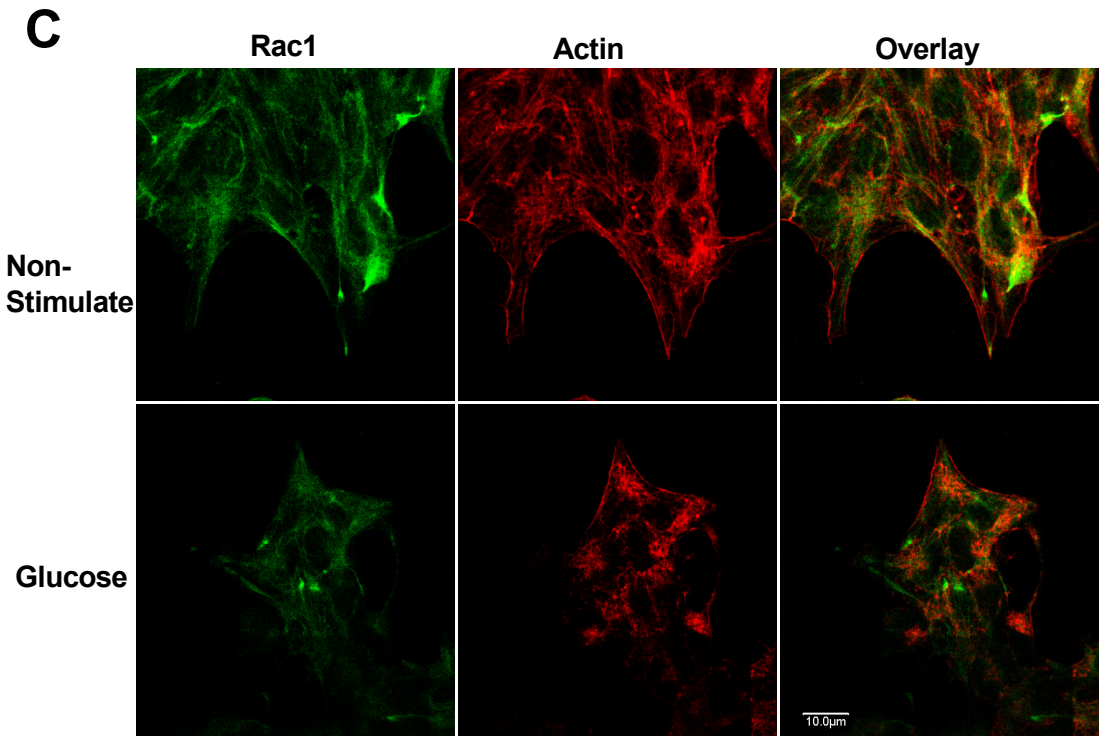

Fig. 5. Glucose stimulation-induced Rac1 translocation did not target to F-actin filaments. Rac1 translocation induced by 15 mM glucose stimulation in INS-1 cells was examined by immunofluorescence staining with FITC conjugated anti-Rac1 monoclonal antibody. F-actin was displayed by rhodamine-phalloidin staining. Control cells (A), N17 transfected cells (B) and V12Rac1 transfected cells (C) were examined by Olympus IX70 Fluoroview 300 laser confocal microscopy. Bar=10 μm.

A4. Glucose- and mastoparan-induced Rac1 translocation to secretory granules in INS-1 cells was inhibited by expression of dominant inhibitory and constitutively active Rac1.

Both glucose and mastoparan could induce Rac1 translocation to membranes from cytosol (Ref. Fig. 3.5, 3.6 and Amin et. al. 2003). To investigate if Rac1 translocation targets to secretory granules beside plasma membrane, the Rac1 translocation in INS-1 cells was detected by immunofluorescence staining and confocal microscopy upon stimulated by either glucose or mastoparan. Both 15 mM glucose and 1 μm

mastoparan stimulation could induce increment of co-localization of Rac1 and the secretory granule marker insulin compared with non-stimulated cells (Fig. 6A). However, in cells expressing N17Rac1, there was partly co-localization of Rac1 and insulin in non-stimulated cells, and glucose and mastoparan stimulation did not augment this co-localization (Fig. 6B). In cells expressing V12Rac1, the signals of co-localization of Rac1 and insulin was stronger than control cells and N17Rac1 cells even in non-stimulated cells, while the glucose and mastoparan stimulation could not significantly further increase the extent of co-localization (Fig. 6C). These results suggested that both glucose and mastoparan could induce Rac1 translocation to secretory granules in INS-1 cells. N17Rac1 and V12Rac1 could localize in secretory granules without stimulation. Their location in secretory granules may attenuate or abolish endogenous Rac1 translocation induced by secretagogues.

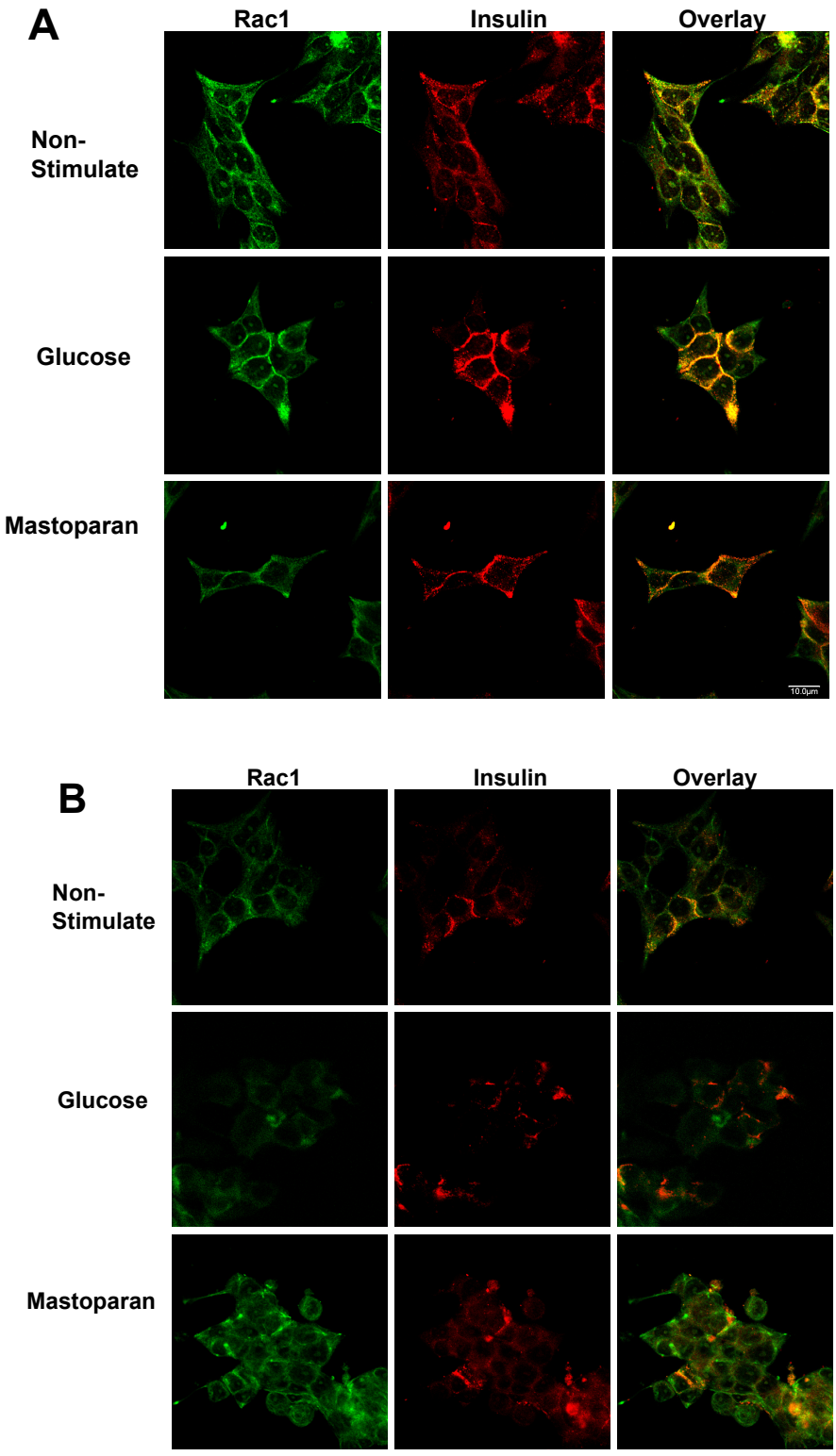

Fig. 6. (Continue to next page)

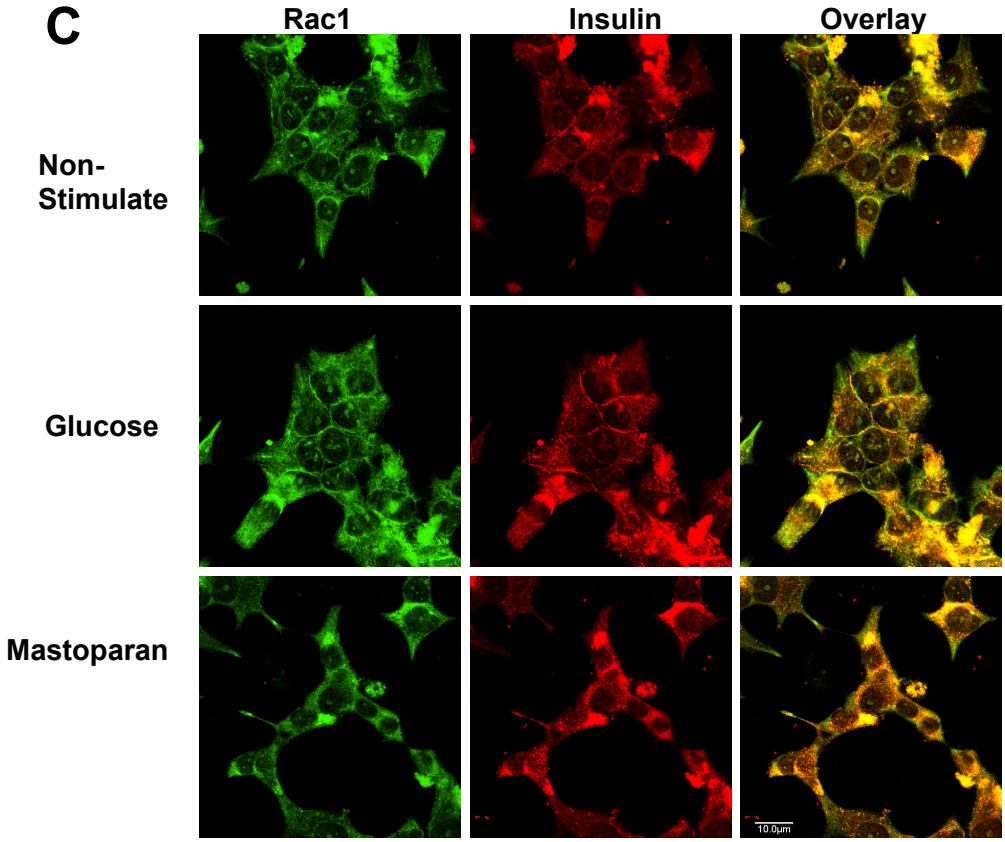

Fig. 6. Glucose and mastoparan induced Rac1 translocation to secretory granules in INS-1 cells. Rac1 translocation induced by 15 mM glucose or 1 μM mastoparan stimulation in INS-1 cells was examined by immunofluorescence staining with FITC-conjugated anti-Rac1 monoclonal antibody. Insulin secretory granules were demonstrated by anti-insulin monoclonal antibody and TRITC-conjugated anti-mouse IgG. Control cells (A), N17 transfected cells (B) and V12Rac1 transfected cells (C) were examined by Olympus IX70 Fluoroview 300 laser confocal microscopy. Bar=10 μm.

A5. Syncollin inhibited stimulated insulin secretion in perifused INS-1 cells

The effect of transfection of complete syncollin gene on insulin secretion was also examined during perifusion of cells. Stimulation of control cells with 15 mM glucose plus 1 μM forskolin for 10 min elicited markedly increase of insulin release while

withdrawal of the stimulants caused fast decline of secretory rates (Fig. 7). Two additional pulses of stimulation also evoked large insulin secretory peaks. In contrast, the stimulated insulin secretion during the three pulses was similarly suppressed in syncollin-transfected cells, although the basal secretory rate was not affected (Fig. 7). The results suggested that expression of syncollin caused impairment of stimulated insulin release may not result from interference with the granule trafficking to a readily releasable pool.

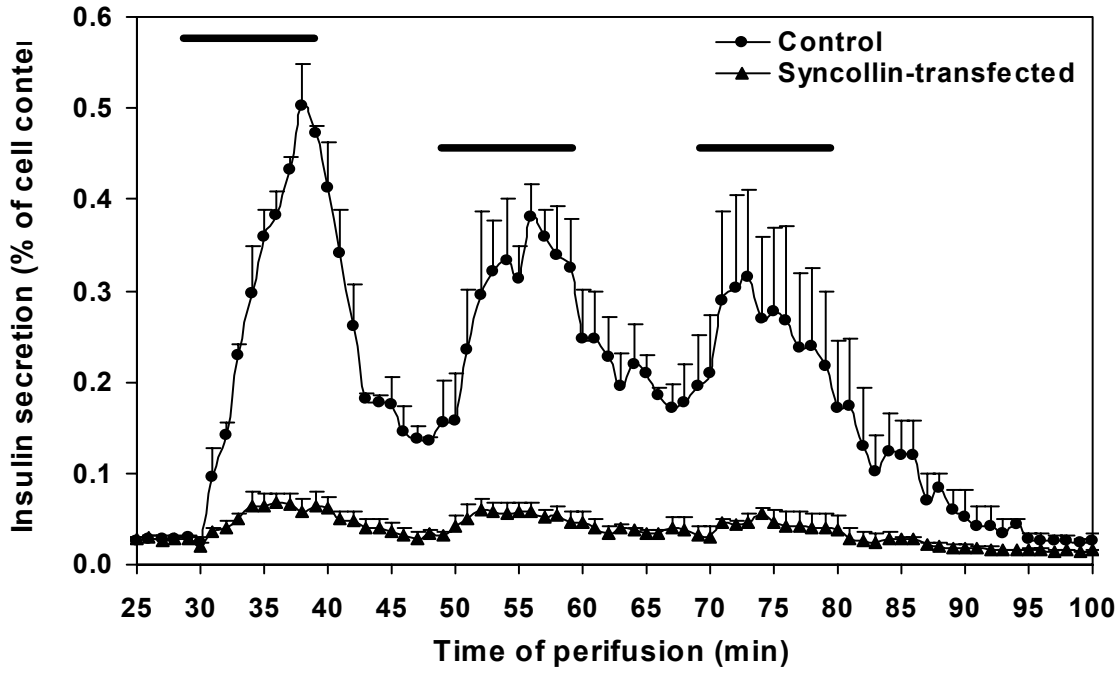

Fig. 7. Perfusion assay for insulin secretion in syncollin transfected INS-1 cells. 2×10^6 cells were perifused in filter chambers for 30 min with KRB containing 2.8 mM glucose for achieving stable basal secretory rates. Thereafter the cells were stimulated with 15 mM glucose plus 1 μM forskolin for 3 pulses each of 10 min. Insulin was measured by RIA. The results of insulin secretion were expressed as percentages of insulin content. Data are the mean ± SEM of 3 observations.

A6. Forskolin-potentiated insulin release was not totally dependent on protein kinase A activation

It is known that activation of protein kinase A (PKA) by cyclic AMP (cAMP) potentiates nutrient-induced insulin secretion from islet β-cells. An increase of intracellular cAMP levels is achieved by stimulation of adenylyl cyclase following the binding of an agonist to its receptor and/or secondary to an elevation of $[Ca^{2+}]_i$. In order to understand to what extent the role of PKA in the regulation of insulin secretion, a potent and selective PKA inhibitor, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H-89) with a K_i of 0.05 μM for PKA (1/10 of other kinases), was applied during 30 min pretreatment and 30 min incubation of insulin-secreting HIT-T15 cells with stimulants (10 mM glucose alone or plus 1 μM forskolin). It is well known that forskolin is able to stimulate adenylyl cyclase directly and thus raise cAMP levels in cells. PKA activity in homogenates from cells treated with H-89 was suppressed in a dose-dependent manner, as measured by using PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay Kit (Promega) (Fig. 8). Forskolin augmented glucose-induced insulin release by ~3 folds. However, 0.3 μM H-89 did not inhibit insulin release induced by either 10 mM glucose alone or combined with forskolin (Fig. 9). Higher doses of H-89 (1-3 μM) reduced the stimulated secretion by ~35% and, under these conditions; forskolin was still capable of significantly potentiating the glucose effect (Fig. 9). Ten μM H-89 could abolish stimulated insulin release. These results suggested that H-89 might also affect insulin secretion via a mode unrelated to PKA inhibition. Therefore, the possible effect of H-89 on $[Ca^{2+}]_i$ was examined by using a fluorescent probe (fura-2). H-89 treatment could reduce the $[Ca^{2+}]_i$ raises induced by glucose as well as by high (40 mM) K^+ (reduction by 22, 30, 46, and 85% at 0.3, 1, 3, and 10 M H-89, respectively), and this

effect was dose-dependent (Fig. 10). Importantly, this inhibitory effect was acute, as demonstrated by the immediate drop of elevated $[Ca^{2+}]_i$ due to high K^+ after adding the agent. In addition, H-89 inhibited K^+-evoked insulin release in a dose-dependent manner closely correlated to its inhibitory effect on $[Ca^{2+}]_i$. These data indicated that H-89 inhibits insulin release mainly by blocking $[Ca^{2+}]$ influx and that an increase of cAMP production by forskolin may also potentiate secretion in a way independent of PKA.

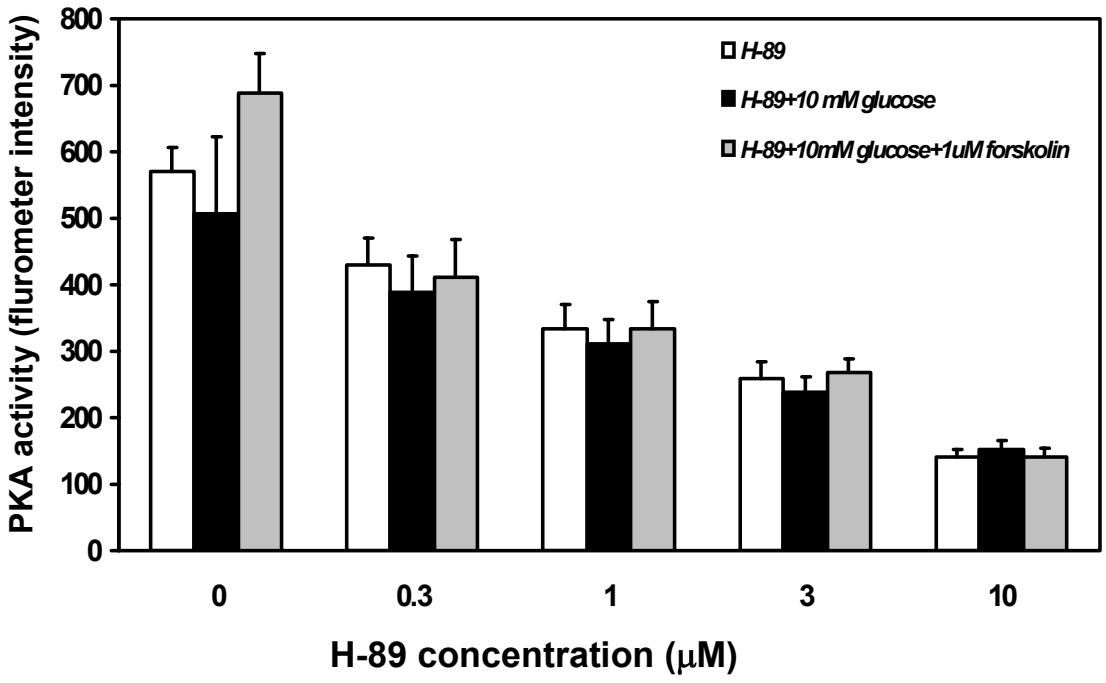

Fig. 8. Protein Kinase A activity under treatment by PKA inhibitor H-89 in HIT-T15 cells. PKA activity in cell homogenates was examined by the product of a fluorescence peptide substrate catalyzed by PKA. The fluorescence of the product was assayed by Perkin-Elmer LS50B luminescence spectrometer. The result of PKA activity was expressed as fluorescence intensity. Data are the mean ± SEM of 4 observations.

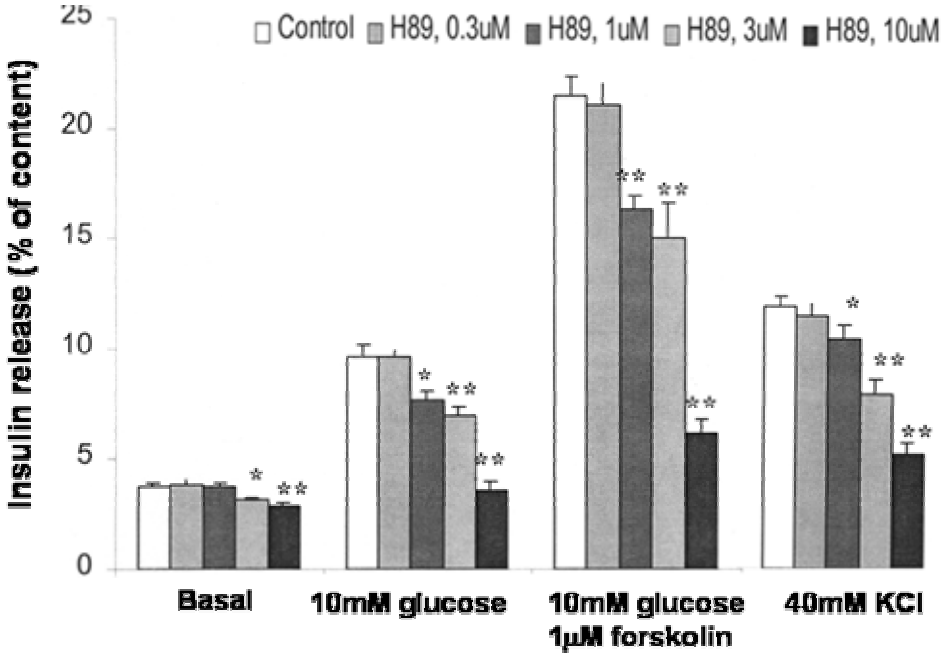

Fig. 9. H-89 inhibited stimulated insulin secretion. HIT-15T cells were pretreated with H-89 for 30 min and then incubated with secretagogues for 30 min in the continuing presence of H-89. Insulin secretion and insulin content were measured by RIA. The values are mean ±SEM of 4 independent experiments in triplicates. * $P<0.05$ and ** $P<0.01$ vs. control.

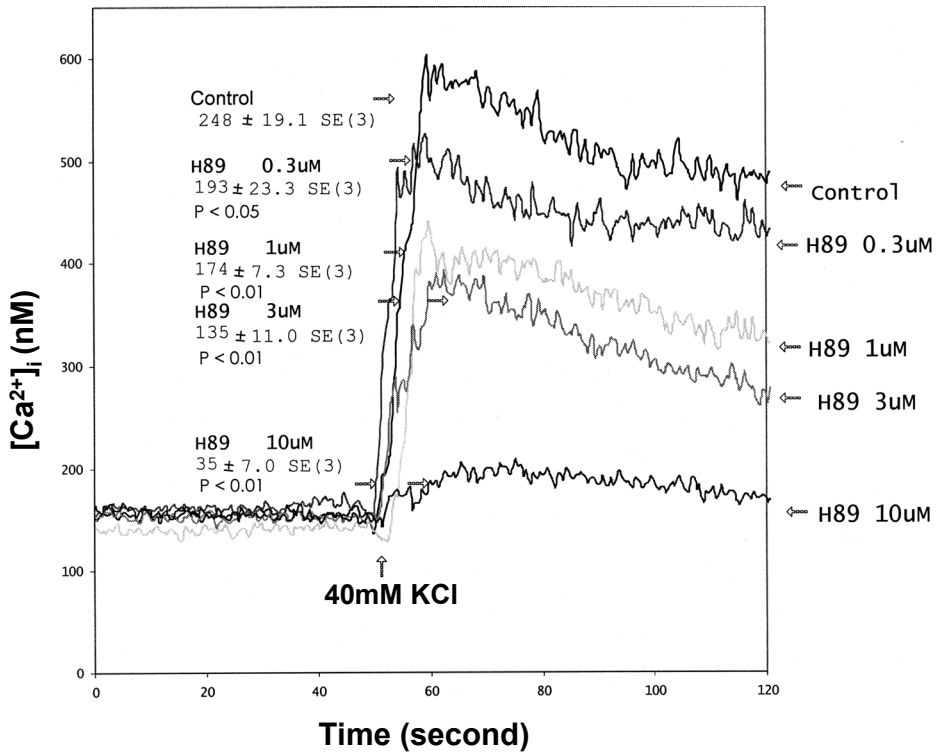

Fig. 10. Effect of H-89 on high K^+ induced $[Ca^{2+}]_i$ raises. $[Ca^{2+}]_i$ in HIT-15T cell suspension was measured by using a fluorescent probe (fura-2). H89 was added 2 min before KCl. All traces are representatives of 3 observations in each case. The indicated values of KCl additions in the figure denote the final concentrations reached in the medium.